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14. ABSTRACT The overall objectives of the study were to understand the mechanism of action of sulfur mustard (HD) on the lung, and ultimately design therapeutic interventions to preserve pulmonary function and life. Under the previous contract (HDTRA 1-07-C-0027) a small proof of principle study investigated the toxicity of inhaled HD. Injury development was monitored in an anesthetized large white pig for 6 hours post exposure and identified dose dependent changes in injury development. The first year of this contract (W81XWH-09-C-0083) was a continuation of the study and extended the model to 12 hours. A reproducible model, representative of human exposure, has been established. Exposure to 100 µg.kg-1 inhaled HD resulted in a significant decrease in oxygenation from 6 to 12 hours post exposure. Pathological examination revealed mucus plugs, areas of edema, inflammatory cell infiltration and necrosis of the tracheal epithelium. The data are consistent with clinical descriptions reported following human exposure to HD (Pechura and Rall., 1993; Hefazi et al., 2005; Balali-Mood & Hefazi 2006). The final phase of the programme was to investigate an appropriate treatment strategy against inhaled HD-induced lung injury in the established model. The thiol compound, N-acetyl-L-cysteine (NAC - Mucomyst™) was chosen due to its anti-oxidant and mucolytic effects, administered via the inhaled route. NAC had been shown to present potential therapeutic benefit against HD-induced lung injury in a small animal model (Anderson D et al; 2000). In our model results indicate physiological benefit following inhaled NAC therapy; further studies are warranted to look at NAC in combination with other beneficial therapies.					
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Introduction

Purpose: The overall objectives of this programme were to attempt to identify the mechanism of action of sulfur mustard (HD) on the lung, and ultimately identify and test therapeutic interventions to preserve pulmonary function and life.

Scope: The aim of this programme was to establish a clinically relevant, reproducible large animal model of inhaled HD exposure. This was conducted in the terminally anesthetized, spontaneously breathing large white pig. Intubation of these animals with HD exposure direct to the lung via the endotracheal (ET) tube better represents human exposure via the nose and mouth when compared with small animal models.

The programme was conducted in 3 discreet phases:

6 h Dose ranging

A dose ranging study was established to identify the doses of HD that produced a range of lung injuries, out to 6 hours post exposure. A number of control animals were also exposed to air demonstrating stable physiology and pathology over a 6 h period, giving confidence in the model. This work was conducted under the previous contract HDTRA 1-07-C-0027.

12 h Toxicology

Secondly, a 12 h toxicology study investigated the physiological and pathological effects of a moderate, but still potentially lethal, dose of HD, taken from the 6 h study. This model allowed an assessment of the toxicology of HD poisoning with clinically relevant physiological parameters in an intensive care setting and established a reproducible model of lung injury following inhaled HD exposure. Assessment of the toxicological profile of HD exposure identified changes consistent with clinical descriptions of human poisoning, allowing confidence to assess potential treatment strategies. This work was conducted under the contract W81XWH-09-C-0083.

12 h Treatment study

Finally, the efficacy of the commercial off the shelf (COTS) drug, N-acetyl-L-cysteine (Mucomyst™; NAC), in ameliorating inhaled HD-induced lung injury was then assessed in the established model. This work was conducted under the contract W81XWH-09-C-0083.

This report summarizes the findings from the 6 h dose ranging, 12 h toxicology and NAC treatment studies. It is supported by two peer reviewed publications (Fairhall *et al.*, 2008, Fairhall *et al.*, 2010) and one paper in preparation (Jugg *et al.*, 2012). Dstl technical reports detailing each of the phases of the study and full data sets are also appended to this document (Dstl/TR50669 and Dstl/TR60201). DTRA Annual reports Mar 10 and 11 should also be referred to.

Major Findings:

6 h Dose ranging

This study demonstrated stable physiology and pathology in air-exposed animals over 6 h, giving confidence that this was an appropriate model in which to investigate the toxicology of inhaled HD.

Exposure to increasing doses of HD caused dose-dependent changes in a number of physiological parameters by 6 h, seen as a decrease in arterial oxygenation and oxyhemoglobin levels, a corresponding increase in reduced hemoglobin levels, as well as an increase in shunt fraction and cardiac output (CO) in HD-exposed animals.

An inhaled dose ($100 \mu\text{g.kg}^{-1}$) of HD was identified which produced a significant and consistent acute lung injury (ALI), which was severe but not lethal at 6 h. This dose was used in the 12 h toxicology study.

12 h Toxicology

A 12 h reproducible model of lung injury following HD exposure was established using an inhaled dose of HD of $100 \mu\text{g.kg}^{-1}$. Assessment of the toxicological profile of HD exposure showed a progression of the changes identified in the 6 h dose ranging study. These changes were consistent with clinical descriptions of human poisoning, allowing confidence to assess potential treatment strategies. The early identification of the mono-sulfoxide β -lyase metabolite of HD, 1-methylsulfinyl-2-[2(methylthio)ethylsulfonyl]ethane] (MSMTESE), in urine has the potential to be a diagnostic and prognostic indicator of HD exposure and treatment efficacy.

12 h Treatment study

In this final study, the ALI resulting from exposure to $100 \mu\text{g.kg}^{-1}$ HD was consistent with that described in the 12 h toxicology study, demonstrating the robustness of the model. Multiple doses of inhaled NAC were an effective treatment for reducing key clinically relevant physiological parameters of inhaled HD-induced lung injury.

Results:

6 h Dose ranging

Normal lung physiology was maintained in air control animals (lying supine and spontaneously breathing) up to 6 h post air exposure. Shunt fraction was significantly increased across all three HD exposed groups (60, 100 and 150 $\mu\text{g.kg}^{-1}$) when compared to air controls at 3 - 6 h post exposure. HD exposed animals were increasingly hypoxemic with respiratory acidosis. Pathological examination revealed necrosis and erosion of the tracheal epithelium in 100 $\mu\text{g.kg}^{-1}$ and 150 $\mu\text{g.kg}^{-1}$ HD exposed groups. The mustard metabolite MSMTESE (an unequivocal marker of HD), was detected in urine from 2 h post exposure. A dose of 100 $\mu\text{g.kg}^{-1}$ HD was chosen to take forward into the toxicology study.

12 h Toxicology

Normal lung physiology was maintained in air control animals up to 12 h post air exposure. In the HD exposed group (100 $\mu\text{g.kg}^{-1}$) there were statistically significant changes in oxygenation parameters. Arterial blood oxygenation (PaO_2) decreased from 6 - 12 h, with an associated decrease in oxyhemoglobin. These animals developed a respiratory acidosis with significant decreases in arterial base excess, arterial bicarbonate and sodium concentrations as well as increased hematocrit. MSMTESE was detected in urine 4 h post exposure with levels increasing until the end of the study. Pathological examination revealed areas of edema, inflammatory cell infiltration and necrosis of the tracheal epithelium in the HD exposed group. These findings are consistent with those seen in clinical descriptions of the early stages of ALI.

Plasma, bronchoalveolar lavage (BAL) fluid and urine samples were analyzed for desmosine, collagenase (matrix metalloproteinase, MMP) activity, neutrophil elastase (NE) activity and myeloperoxidase (MPO) activity. There was evidence of sulfur mustard-induced neutrophil activation in the lung and elastase-mediated breakdown of the matrix protein elastin, measured as desmosine in urine. This provided evidence for the presence of activated neutrophils in the lung.

12 h Treatment study

HD control animals showed physiological changes consistent with those reported in the 12 h toxicology study, demonstrating that the model is robust. HD controls developed an ALI as shown by decreased arterial blood oxygenation, bicarbonate and base excess, with increased arterial blood pCO_2 when compared to baseline values. Animals were increasingly hypoxemic with respiratory acidosis. Shunt fraction was significantly increased at 12 h compared to baseline values and the significantly increased heart rate reflected the heart compensating for this. Terminal BAL fluid analysis showed an increased number of neutrophils recruited into the lungs, and small increase in protein concentration, 12 h post exposure compared with air controls from the 12 h toxicology study. An increase in the MSMTESE metabolite was apparent in urine from 4 h, before rapidly increasing from 6 h post exposure.

Multiple inhaled doses of NAC significantly improved arterial blood oxygenation, reduced heart rate and shunt fraction, as well as other markers of lung injury (significantly reduced neutrophil numbers and protein concentration in terminal BAL fluid), compared with HD controls. Pathology

revealed damage was confined to the areas below the ET tube in both groups, with areas of inflammation, epithelial sloughing and mucus production. These effects were visually less pronounced in the NAC treated animals. There was no significant difference in the amount of MSMTSE detected in the urine of the NAC treated group compared to HD controls. Analysis of lung homogenates for thiols found significantly higher levels in NAC treated animal lungs than HD controls.

A significant increase in urinary desmosine was noted at 4 hours post exposure in HD control animals which was prevented following NAC treatment. However, there was no difference between groups over the course of the study. No significant differences were detected in BAL fluid desmosine levels between groups. Terminal BAL fluid MPO, NE and MMP activity were also not significantly different between groups however there was a downward trend for all markers in the NAC treated animals.

Significance:

6 h Dose ranging

This study demonstrated that terminally anesthetized, spontaneously breathing large animals displayed stable physiology and pathology 6 h post air exposure. Development of a novel exposure system allowed delivery of HD vapor direct to the lung (via the ET tube) better representing human exposure via the nose and mouth. Inhaled HD-induced lung injury manifest as reduced oxygenation and increased shunt fraction with respiratory acidosis at 6 h. Pathology revealed necrosis and erosion of the tracheal epithelium. An inhaled dose of HD ($100 \mu\text{g} \cdot \text{kg}^{-1}$) was identified to be taken forward into the 12 h toxicology study.

12 h Toxicology

Assessment of the toxicological profile of HD exposure (inhaled dose $100 \mu\text{g} \cdot \text{kg}^{-1}$) identified changes consistent with clinical descriptions of human poisoning, allowing confidence in assessing potential treatment strategies in this model.

The results provide evidence of HD-induced neutrophil activation in the lung and elastase-mediated breakdown of the matrix protein, elastin. Breakdown of lung matrix by MMPs may contribute to HD induced lung injury. Urinary desmosine may be a useful indicator of lung injury, however this may be non-specific. These results are in agreement with previous studies indicating that MMPs play a role in HD induced lung injury and increase our confidence in the model (Anderson *et al.*, 2009; Kehe *et al.*, 2009 and Calvet *et al.*, 1999).

12 h Treatment study

Multiple inhaled doses of NAC significantly improved arterial blood oxygenation parameters, reduced heart rate and shunt fraction and reduced neutrophil numbers and protein concentration in terminal BAL fluid, compared with HD controls. Pathological damage was visually less pronounced in the NAC treated animals. Analysis of lung homogenates found lung tissue thiol concentrations were significantly increased in the NAC treated animals when compared to HD controls.

NAC delayed the early increase in urinary desmosine levels following HD exposure, reflecting a dampening effect on elastin degradation. The decrease in number of neutrophils recruited into the lungs was reflected by decreases in markers of neutrophil activation (NE, MPO and MMP), however these effects were not significant.

This is the first time that any treatment has been shown to be efficacious for HD-induced lung injury in two animal species (Anderson *et al.*, 2009). As NAC is a COTS drug it could be rapidly transitioned into front line medical care.

Further studies should now be conducted to investigate the efficacy of multiple inhaled therapies (incorporating NAC) to further improve the physiological indices in HD-induced ALI.

1.0 Body

The mechanism of action by which inhaled HD causes effects on the lung are still poorly understood. Although best known as a vesicating agent, the majority of deaths associated with mustard exposure involve some degree of pulmonary damage. The involvement of lung injury is a major factor in mustard related mortality. The respiratory effects of exposure to inhaled HD in humans are well recorded (Vedder E, 1925).

Currently no medical countermeasures exist to treat HD-induced ALI, with treatment being supportive, often in an intensive care situation, which places a high burden on civilian/military medical staff. These healthcare systems could be easily overwhelmed in a mass casualty event. Hence, there is an ongoing requirement to find efficacious treatments for chemically-induced ALI.

Previous work under contract HDTRA 1-07-C-0027 established a large animal model of chemically-induced lung injury. The terminally anesthetized, instrumented pig was used to characterize the range of injuries to the lung, over a 6 h time period caused by a range of doses of inhaled HD (60, 100, 150 $\mu\text{g.kg}^{-1}$ inhaled dose). This model used a novel exposure system (Fairhall *et al.*, 2008) which better represented the human exposure, since humans breathe via both the nose and mouth. Small animals are obligate nasal breathers and therefore can show subtle differences in injury pathology if they are not intubated. This model is a significant improvement on the use of small animals, since it allows the use of a large number of human physiological monitoring systems to fully characterize the effects of HD on the animal. A dose response was established with dose dependent changes being identified (Fairhall *et al.*, 2010).

The model was then used to further elucidate the effects of inhaled HD out to 12 h (contract W81XWH-09-C-0083). Animals were exposed to a dose of HD vapor identified from the 6 h dose ranging study as one that might cause a severe, but non lethal ALI by 12 h (100 $\mu\text{g.kg}^{-1}$). Assessment of the toxicological profile of HD exposure identified changes consistent with clinical descriptions from human poisoning, allowing confidence to assess potential treatment strategies.

Both studies identified the early presence of the urinary HD metabolite and unequivocal marker of HD poisoning, MSMTESE, which has the potential to be a diagnostic and prognostic indicator of HD exposure and treatment efficacy.

The efficacy of inhaled NAC as a treatment for HD-induced lung injury was then assessed. Animals were exposed to inhaled HD vapor alone (HD controls) or, post exposure, were treated with inhaled NAC. HD controls showed physiological and pathological changes consistent with those seen in the 12 h toxicology study, demonstrating the robustness of the model. Treatment with multiple inhaled doses of NAC significantly improved oxygenation, cardiovascular and calculated parameters whilst reducing neutrophil and protein infiltration to the lungs. Lung tissue thiol concentrations were significantly increased in the NAC treated animals when compared to HD controls. This effect was independent of section (left or right lung), or lobe (apical, medial or caudal). A significant increase in urinary desmosine was noted 4 hours post exposure in HD control animals which was prevented by NAC. However, there was no difference between groups over the course of the study. No significant differences were detected in BAL fluid desmosine levels between groups. Terminal BAL fluid MPO, NE and MMP

activity were also not significantly different between groups however there was a downward trend for all markers in the NAC treated animals.

These results will contribute to an evidence based strategy to military and civilian medical personnel for the treatment of chemically-induced lung injury.

1.1 Experimental Details

Full details of all of the experiments carried out as part of this programme can be found in the appended technical reports (Dstl/TR50669 and Dstl/TR60201). Below is a brief summary of the methodology followed for each of the studies.

For all of the studies animals (approx. 50 kg) were surgically prepared (under terminal anesthesia) so that access could be gained (via implanted cannulae) to arterial and venous lines to obtain blood samples, administer intravenous anesthesia and fluids and measure cardiovascular parameters e.g. PiCCO. The bladder was also catheterized to obtain hourly urine samples. Animals were exposed to HD vapor (or air) as previously described (Fairhall *et al.*, 2008). In brief, the animals were maintained supine and allowed to breathe spontaneously while baseline measurements were taken for 1 h. They were then connected to the exposure apparatus via a polytetrafluoroethylene (PTFE)-lined ET tube, a size 2 Fleisch pneumotachograph (for measurement of respiratory parameters) and a sample port for the duration of the exposure which delivered the HD vapor (or air) direct to the lung. The concentration of HD generated for each group was kept constant, as the Porton vapour generators (PVGs) are slow to equilibrate following temperature change, and the desired inhaled dose reached by varying the length of exposure. Effort was also made to maintain the animals' minute volumes between 6 and 8 L.min⁻¹ by controlling the dose of anesthetic. The inhaled dose was then estimated every minute during the exposure, and the exposure terminated when the inhaled dose reached the desired value. Thus, whilst the exposure duration was planned to be 10 min, in practice it varied to account for uncontrollable variations in the animal's minute volume from 7 L.min⁻¹.

6 h Dose ranging

Anesthetized, spontaneously breathing large white pigs were exposed to inhaled doses of HD vapor of 60 µg.kg⁻¹ (n = 5, Low), 100 µg.kg⁻¹ (n = 5, Medium) or 150 µg.kg⁻¹ (n = 5, High), or to air (n = 4), and monitored for 6 h (Fairhall *et al.*, 2010). Cardiovascular and respiratory parameters were recorded continuously throughout the experiment. Blood and terminal BAL fluid were collected to allow blood gas and hematology analysis, and to assay for lung inflammatory cells and mediators. Urine was collected and analyzed for HD metabolites. Tissue samples were taken *post mortem* and processed for histopathological analysis.

12 h Toxicology

Anesthetized, spontaneously breathing large white pigs were exposed to HD vapor at 60 µg.kg⁻¹ (n = 3, Low), 100 µg.kg⁻¹ (n = 7, Medium), or to air (n = 6), and monitored for 12 h. Animals exposed to 60 µg.kg⁻¹ showed little change in pathophysiology from air-exposed controls at 12 h. Therefore a decision was taken to continue with the 100 µg.kg⁻¹ dose in order to meet the objectives of this programme. Cardiovascular and respiratory parameters were recorded

continuously throughout the experiment. Blood and terminal BAL fluid were collected to allow blood gas and hematology analysis, and to assay for lung inflammatory cells and mediators. Urine was collected and analyzed for HD metabolites. Tissue samples were taken *post mortem* and processed for histopathological analysis and genomic analysis. Genomic analysis (to identify potential targets for drug interaction) was carried out at the United States Army Medical Research Institute for Chemical Defense (USAMRICD).

12 h Treatment study

Anesthetized, spontaneously breathing large white pigs were exposed to HD vapor ($100 \mu\text{g.kg}^{-1}$) determined using Fourier Transform Infrared (FTIR) technology (Gasmeter™) (Dstl/TR6020, Annex A) and monitored for 12 h. Animals were randomly assigned to either HD control (HD alone; n=6) or HD treated (HD followed by inhaled NAC (1 ml of 200 mg.ml^{-1} solution of Mucomyst™ at + 30 min, 2, 4, 6, 8 and 10 h post exposure (estimated total dose delivered 1200 mg; n=8)) groups. Cardiovascular and respiratory parameters were recorded continuously throughout the experiment. Blood and terminal BAL fluid were collected to allow blood gas and hematology analysis, and to assay for lung inflammatory cells and mediators. Urine was collected and analyzed for HD metabolites. Tissue samples were taken *post mortem* and processed for histopathological, genomic and thiol analysis.

Tissue and Sample analysis

Desmosine, Collagenase, Neutrophil Elastase (NE) and Myeloperoxidase (MPO).

Analysis of desmosine, collagenase (MMP) activity, NE activity and MPO activity were carried out at the University of Portsmouth using commercially available reagents (Dstl/TR50669 Annex A; Dstl/TR60201 Annex D). Urine samples were analyzed for desmosine and isodesmosine using a competitive ELISA (Elastin Products company, Missouri, USA). Plasma and terminal BAL fluid samples were analyzed for collagenase activity using an assay kit obtained from Molecular Probes, Rijnsburgerweg, Netherlands. NE and MPO activity were assessed in terminal BAL fluid samples using commercially available reagents.

Enzyme Linked Immunosorbent Assays (ELISAs)

Mediators (IL-1 β , IL-6, IL-8 and TNF α) were measured in terminal BAL fluid samples using commercially available standard quantitative porcine ELISA kits (R&D Systems, Abingdon, UK).

Genomics

12 h toxicology study:

An analysis of the transcriptomic changes in HD exposed cells in pig lung was performed at USAMRICD using microarray (Affymetrix Porcine) data from 28 lung samples from pigs exposed to different conditions: air, low dose of HD, and medium dose of HD. Within each exposure condition, RNA was extracted from three different lobes; caudal, apical, and median. A 1-way ANOVA test comparing the difference in gene expression from different exposure conditions was performed on microarray data from these samples in Partek Genomic Suite v 6.4, which resulted in 126 significant probes after a Bonferroni test correction was performed on the p-values. The 126 significant probes from the aforementioned ANOVA were translated into their corresponding probe set IDs from the Affymetrix Human U133+2.0 chip and uploaded

into Ingenuity Pathway Analysis (IPA). Genes that did not have a corresponding gene in *Homo sapiens* were not included in analysis leaving a total of 113 ID's, 111 of which were correlated to genes in the human genome. A Core Analysis was performed on this data set.

Urine adducts

Urine aliquots were analyzed for MSMTESE using a modified method from that published by Read and Black (2004). This was modified by use of an alternative C18 liquid chromatography (LC) column (ACE 3 C18HL, 150 mm x 2.1 mm; Hichrom Ltd, Reading, UK). Calibration was performed using urine (from pigs exposed to air in the dose ranging study) that had been spiked with MSMTESE over the range 0.1 to 1000 ng.ml⁻¹. Deuterated (D6) MSMTESE was used as internal standard for quantitation.

Thiols

Thiols were quantitated using the Molecular Probes Thiol and Sulfide Quantitation Kit (Life Technologies Ltd., Paisley, UK). Thiols reduce disulfide-inhibited papain to release active enzyme. Papain activity is measured using its reaction with *N*-benzoyl-L-arginine *p*-nitroanilide to release *p*-nitroaniline. The absorbance of *p*-nitroaniline is measured at a wavelength of 412 nm. Calibrations are performed against L-cysteine and the linear range is approximately 0.2 to 1.5 µM. Samples with thiol concentrations exceeding 1.5 µM were diluted prior to re-analysis.

Histopathology.

Pathology samples were sent to the Veterinary Laboratories Agency (VLA) for an independent pathologist to score the slides for pathological changes. A scoring system was developed to assess the lung pathology, based on: bronchiolar epithelial degeneration and necrosis; hypertrophy/hyperplasia of bronchiolar epithelium; cell debris in the bronchiolar lumen; perivascular (and peribronchiolar) edema, fibrin and cell infiltrates; alveolar edema and fibrin; alveolar cell infiltrates; interstitial (septal) edema; hemorrhages. Parameters were evaluated as follows: 0: Not observed; 1: Mild; 2: Moderate; 3: Severe. The full reports, including injury scoring, are contained in Annex E (6 h) and Annex F (12 h) of Dstl/TR50669 and Annex B (Treatment study) of Dstl/TR60201.

1.2 Statistical Analysis

6 h Dose ranging

Samples were taken at three points pre-exposure. The values obtained at these time-points were averaged and are presented here as a single baseline data point (t=0). The data for each recorded parameter were grouped into one of two categories; 0 - 3 hours and 3 - 6 hours and were checked for normality using an Anderson-Darling test. Data that was not normally distributed was log transformed where possible. The area under the curve (AUC) was then calculated for each parameter within each category. Comparisons of the AUC were then made between the control group and HD treatment groups (60, 100 and 150 µg.kg⁻¹) using either a 2-sample t-test or, for non-parametric data, a Mann-Whitney U-test. Data were expressed as mean (± SEM) and p values <0.05 were considered significant. For statistical analysis of inhaled dose, cytokine and MSMTESE levels, cell differentials and lung wet weight: body weight

(LWW:BW) and lung wet weight: dry weight (LWW:DW) ratio determinations, Bartlett's test was used to assess whether equal variance could be assumed and normality assessed using normal probability plots. Two sample t-tests and analysis of variance (ANOVA) were then carried out. For non-parametric data, Kruskal-Wallis tests were adopted.

12 h Toxicology

Data are expressed as mean \pm SEM. Bartlett's test was used to determine whether the variability of the data was consistent between the air controls and HD exposed groups as well as whether it was consistent over time. The data were investigated at different time points (3, 6, 9 and 12 hours post exposure) to test for differences between groups (Air ▲, HD exposed ▼). Normal probability plots were used to decide whether the assumption of a Normal Distribution, and hence a parametric test, was appropriate for the data. A two-sample t-test was used to compare the average data values for the two groups.

12 h Treatment study

Differences between the HD control (HD alone ●) and NAC treated (HD + NAC ■) groups were investigated at 3, 6, 9, and 12 h post exposure. The average data values between these two groups at individual time points were tested using a two-sample t-test. Assessment of the distribution of the data was performed in order to justify the use of the two-sample t-test. If there was evidence against the data following a normal distribution, a non-parametric alternative (Wilcoxon) was used. Tissue thiol concentrations were analysed using a three way ANOVA with repeated measures (treatment effect; section effect (left or right lung); or lobe effect (apical, medial or caudal)).

1.3 Results

Full results can be found in each of the appended Dstl technical reports (Dstl/TR50669 and Dstl/TR60201). The important highlights are described below.

6 h Dose ranging

Achieved inhaled doses for HD exposures were not significantly different to target inhaled doses demonstrating the excellent reproducibility of the exposure system.

Animals exposed to air or $60 \mu\text{g.kg}^{-1}$ HD survived to 6 h with normal arterial oxygenation and pathology. Animals exposed to an inhaled dose of $100 \mu\text{g.kg}^{-1}$ HD had significantly reduced arterial blood oxygenation from 3 to 6 h post exposure compared to air controls ($p = 0.035$) which was reflected by reduced oxyhemoglobin levels ($p = 0.03$). There was also a significant reduction in arterial blood bicarbonate concentration in HD exposed animals at 0 to 3 h when compared to air controls ($p = 0.016$). However, pH remained stable throughout the 6 h study in both HD exposed animals and air controls. The tracheal epithelium of HD exposed animals revealed dose dependent increases in severity of HD-induced damage, with edema localized to the lamina propria. There was also evidence of neutrophil infiltration throughout the mucosa and submucosa. Lower airway damage was mild with evidence of cellular debris in bronchiolar regions.

Two doses (60 and $100 \mu\text{g.kg}^{-1}$) were taken forward into the 12 h toxicology study with the aim of identifying a dose which caused a severe but non lethal injury after the longer time period.

12 h Studies (Toxicology and Treatment study)

1.3.1 Survival

In the 12 h toxicology study all air control animals and all animals exposed to $60 \mu\text{g.kg}^{-1}$ HD vapor survived to 12 h. Three animals died in the $100 \mu\text{g.kg}^{-1}$ HD group, two of which died due to obstructions in the ET tube at 5.5 and 6.5 h. The third animal died at 10.5 hours from HD-induced lung injury. The two early deaths due to obstructions in the ET tube led to the implementation of a suctioning protocol in all further studies whereby debris within the ET tube was suctioned hourly from 6 h. This was due to the inflexible nature of the lined ET tube which prevented the animal from clearing debris by coughing.

For the treatment study all animals were exposed to a target inhaled dose of $100 \mu\text{g.kg}^{-1}$ HD vapor. The achieved mean inhaled dose was $99.8 \pm 1.07 \mu\text{g.kg}^{-1}$ which was not significantly different from the target inhaled dose. One animal in the HD control group died of HD poisoning within the 12 h study period. All animals exposed to HD and treated with NAC survived to the end of the study.

1.3.2 Physiology and hematology

Animals exposed to $60 \mu\text{g.kg}^{-1}$ HD vapor did not develop a significant injury, therefore for the purpose of the remainder of this report the results will focus on animals exposed to $100 \mu\text{g.kg}^{-1}$ HD from the 12 h toxicology study.

In the 12 h toxicology study, air control animals maintained arterial blood oxygenation throughout. In HD exposed animals there was a significant decrease in oxygenation at 6, 9 and 12 hours ($p<0.05$) when compared to the air control group (Figure 1).

In the 12 h treatment study, arterial blood oxygenation was significantly decreased in the HD control animals at 12 h post exposure ($\dagger\dagger\dagger p<0.001$) compared to baseline values. HD control animals had significantly reduced arterial blood oxygenation at 12 h when compared to the NAC treated animals ($\#p<0.05$ - Figure 1) with a concomitant significant increase in arterial blood carbon dioxide levels ($*p<0.05$ – see Dstl/TR60201). NAC treated animals maintained close to normal oxygenation levels throughout the study.

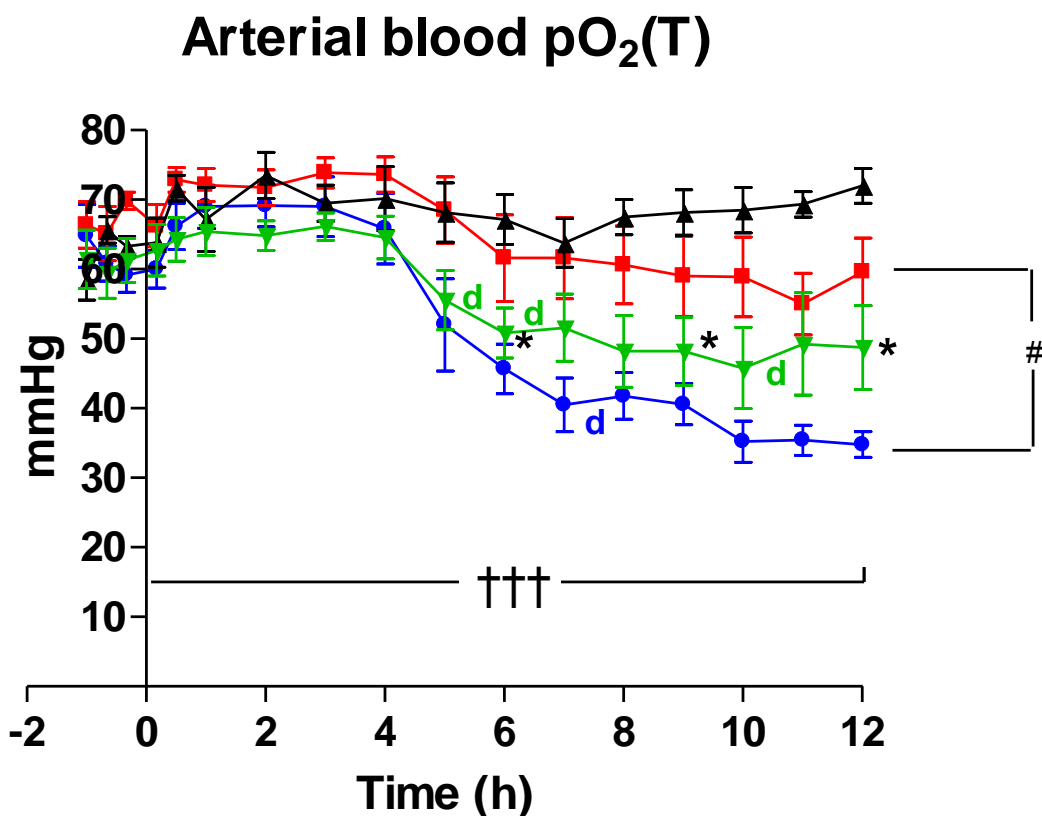


Figure 1. Changes in arterial blood oxygenation following exposure to air (▲ - 12 h tox; $n=6$), 100 $\mu\text{g.kg}^{-1}$ HD (▼ - 12 h tox; $n=7$ and ● - HD Controls; $n=6$), or NAC treated (■; $n=8$). Data are expressed as mean \pm SEM. *Significant difference between air and the HD exposed (▼) at 6, 9 and 12h ($p<0.05$). # $p<0.05$ indicates significant difference between HD control and NAC treated groups at 12 h. $\dagger\dagger\dagger p<0.001$ indicates the statistical significance of changes from baseline levels in the HD control group at 12h. d' represents a death in the associated group.

In the 12 h toxicology studies air control animals maintained arterial blood oxyhemoglobin levels throughout. There were significant decreases in the HD exposed group at 6, 9 and 12 hours post exposure when compared to the air control group (Figure 2).

In the 12 h treatment study, arterial blood oxyhemoglobin levels were significantly decreased in the HD control animals at 12 h post exposure (††† $p < 0.001$) compared to baseline values (Figure 2). There was a concomitant increase in reduced hemoglobin (RHb) levels (full data set presented in DstI/TR60201). The NAC treated group maintained arterial blood oxyhemoglobin at baseline levels throughout. NAC treated animals were significantly different from HD controls for oxyhemoglobin and RHb at 12h ($\#p < 0.05$).

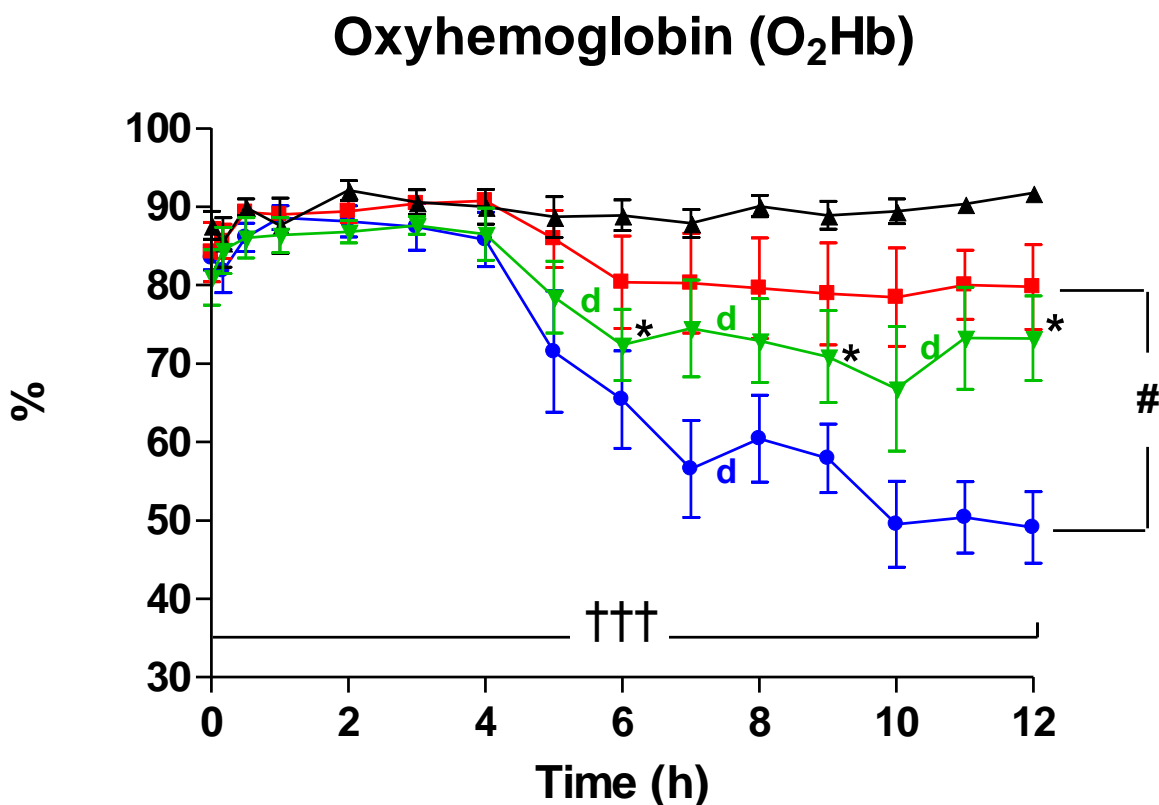


Figure 2. Changes in arterial blood oxyhemoglobin levels following exposure to air (▲ - 12 h tox; $n=6$), 100 $\mu\text{g.kg}^{-1}$ HD (▼ - 12 h tox; $n=7$ and ● - HD Controls; $n=6$), or NAC treated (■; $n=8$). Data are expressed as mean \pm SEM. *Significant difference between air and the HD exposed (▼) at 6, 9 and 12h ($p < 0.05$). # $p < 0.05$ indicates significant difference between HD control and NAC treated groups at 12 h. ††† $p < 0.001$ indicates the statistical significance of changes from baseline levels in the HD control group at 12h. 'd' represents a death in the associated group.

Shunt fraction for air control animals in the 12 h toxicology study was maintained at normal baseline levels (Figure 3). The shunt fraction of the HD exposed group was significantly increased at 6 hours post exposure ($*p < 0.05$).

In the 12 h treatment study shunt fraction was significantly increased at 12 h post exposure in the HD control group when compared to baseline levels (††† $p<0.001$) and when compared to NAC treated animals at the same time point (# $p<0.05$). The NAC treated group maintained shunt fraction at near normal baseline levels (Figure 3).

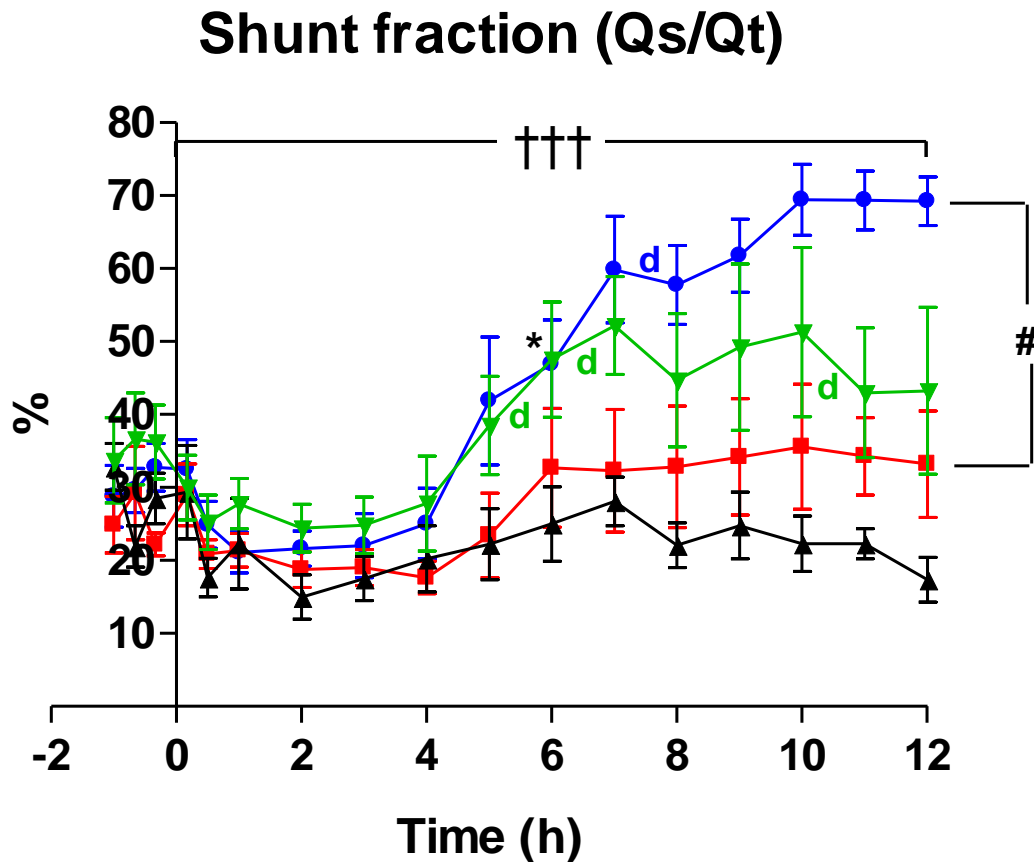


Figure 3. Changes in shunt fraction following exposure to air (▲ - 12 h tox; n=6), 100 µg.kg⁻¹ HD (▼ - 12 h tox; n=7 and ● - HD Controls; n=6), or NAC treated (■; n=8). Data are expressed as mean ± SEM.

*Significant difference between air and the HD exposed (▼) at 6h ($p<0.05$). # $p<0.05$ indicates significant difference between HD control and NAC treated groups at 12 h. ††† $p<0.001$ indicates the statistical significance of changes from baseline levels in the HD control group at 12h. 'd' represents a death in the associated group.

Figure 4 shows a statistically significant decrease in the arterial blood base excess of HD exposed animals from the 12 h toxicology study at 3, 6, 9 and 12 hours when compared to air controls (* $p<0.05$). There was also a significant decrease in the bicarbonate levels in the HD exposed group at 3, 6, 9 and 12 hours post exposure when compared to air controls (* $p<0.05$). This was not reflected by a change in the pH of any of the HD exposed animals, which

remained stable throughout and was not statistically significantly different from air controls (data for bicarbonate and pH presented in Dstl/TR50669).

In the 12 h treatment study arterial blood pH was significantly reduced in the HD control and increased for NAC treated animals at 12 h compared to baseline values ($\dagger\dagger p < 0.01$, $^{\wedge}p < 0.01$). However, there was no significant difference between the HD control and NAC treated groups over the course of the study (Dstl/TR60201).

Bicarbonate concentration was significantly decreased in HD controls at 12 h when compared to baseline values, suggesting a respiratory acidosis in these animals ($\dagger p < 0.05$) which was reflected in the base excess ($\dagger p < 0.05$, Figure 4). Bicarbonate concentration was significantly lower in the HD control animals compared to the NAC treated animals at 12 h ($*p < 0.05$). NAC treated animals maintained normal bicarbonate levels, and hence base excess, throughout the study.

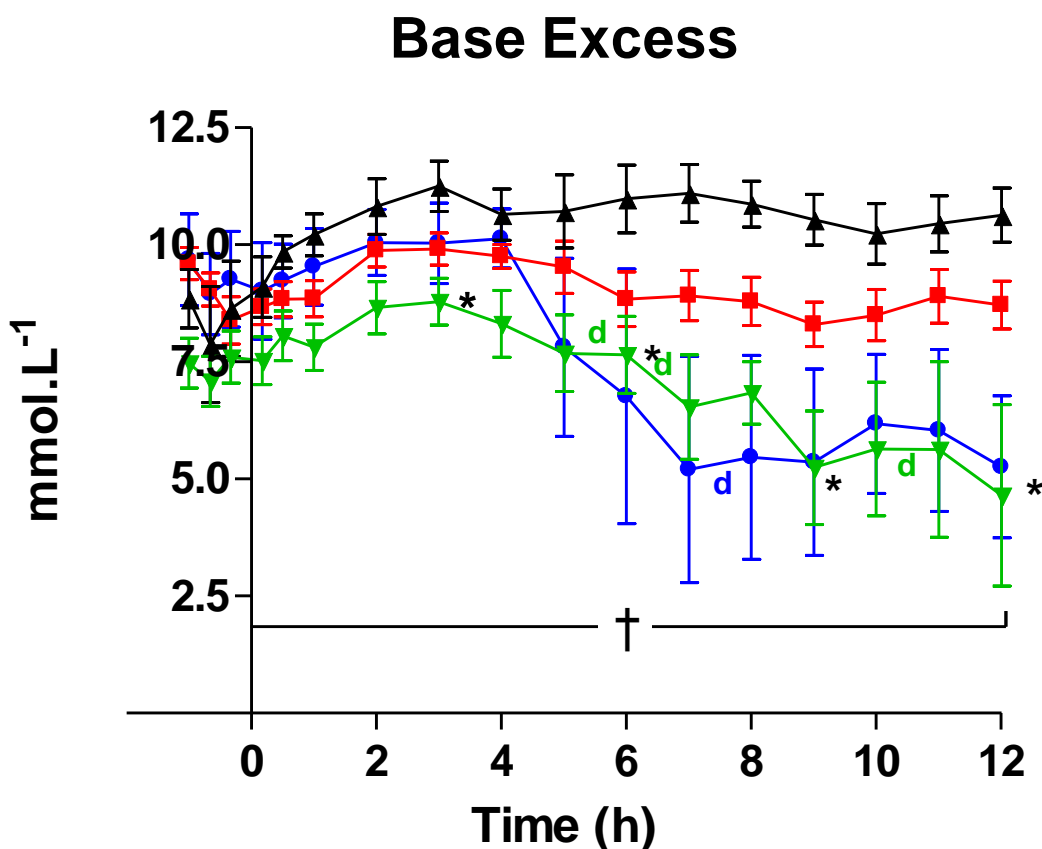


Figure 4. Changes in arterial blood base excess following exposure to air (▲ - 12 h tox; n=6), 100 $\mu\text{g.kg}^{-1}$ HD (▼ - 12 h tox; n=7 and ● - HD Controls; n=6), or NAC treated (■; n=8). Data are expressed as mean \pm SEM. *Significant difference between air and the HD exposed (▼) at 3, 6, 9 and 12 h ($p < 0.05$). $\dagger p < 0.05$ indicates the statistical significance of changes from baseline levels in the HD control group at 12h. 'd' represents a death in the associated group.

1.3.3 ***Tissue and Sample analysis***

Desmosine, Collagenase, Neutrophil Elastase (NE) and Myeloperoxidase (MPO).

In the 12 h toxicology study there was a significant increase in urinary desmosine levels post HD exposure compared to air controls. BAL fluid desmosine concentrations correlated significantly with terminal BAL fluid neutrophil numbers, both as a percentage of total cells and absolute numbers of neutrophils. There was no correlation in urinary desmosine with BAL fluid biomarkers (neutrophil numbers, IL-8, NE, MPO or MMP) or plasma MMP activity in the HD exposed group. There were no significant differences in BAL fluid MMP, MPO or NE activity, although trends towards an increase in NE and MPO activity (markers of neutrophil activation) could be seen between groups. Plasma collagenase activity did not differ following exposure to HD at any time point or following air exposure (Dstl/TR50669).

In the treatment study a significant increase in urinary desmosine was noted at 4 h post exposure in HD control animals (Dstl/TR60201, Annex D). NAC treated animals prevented the early increase in urinary desmosine, however, there was no difference between groups over the course of the study. No significant differences were detected in terminal BAL fluid desmosine levels between groups. Terminal BAL fluid MPO, NE and MMP activity were also not significantly different between groups however there was a downward trend for all markers in the NAC treated animals.

Enzyme Linked Immunosorbent Assays (ELISAs)

ELISA analysis from the 12 h toxicology study are reported in Dstl/TR50669.

ELISA analysis for the NAC treatment study revealed an inconsistent response to the presence of the inflammatory mediators, IL-1 β , IL-6, IL-8 and TNF α across all animals in terminal BAL fluid (Dstl/TR60201, Annex E). Annex E to Dstl/TR60201 shows the comparison of cytokine production for the HD controls and NAC treated groups. Whilst it appears that the NAC may have a dampening effect on cytokine production (IL-6 and IL-8), the lack of reproducibility across animals in the HD control group means that no conclusion can be supported.

Genomics

For the 12 h toxicology study:

Using the list of genes from the data set and Pathway Explorer function in IPA, two pathways were generated that showed genes significant in apoptosis and inflammation from the analysis. Summaries were generated from these pathways, which included the list of drugs that have been noted to affect the pathway's constituent genes. As a result of a small sample size and little variation in gene expression observed across lobes, a smaller set of genes (111) was analyzed. Of those genes analyzed, core analysis showed little overlap in canonical pathways; however, an evident overlap can be seen in their regulation of apoptosis and inflammation. Based on the two generated pathways, potential drug targets may be found.

Sample analysis for the NAC treatment study is awaited.

Urine adducts

HD exposed animals from the 12 h toxicology study showed an increase in MSMTESE levels, with approximately 0.1 ng.ml^{-1} detected as early as 2 hours post exposure, increasing substantially from 6 hours onwards (Figure 5).

In the treatment study MSMTESE could be detected from 4 h in the urine of both HD controls and NAC treated groups before increasing substantially from 6 h. Although the time profile of MSMTESE was similar to the 12 h toxicology study, the concentrations of MSMTESE obtained in the 12 h treatment study were much higher. There was no significant difference in the amount of MSMTESE present in the urine over time between the HD control and NAC treated groups.

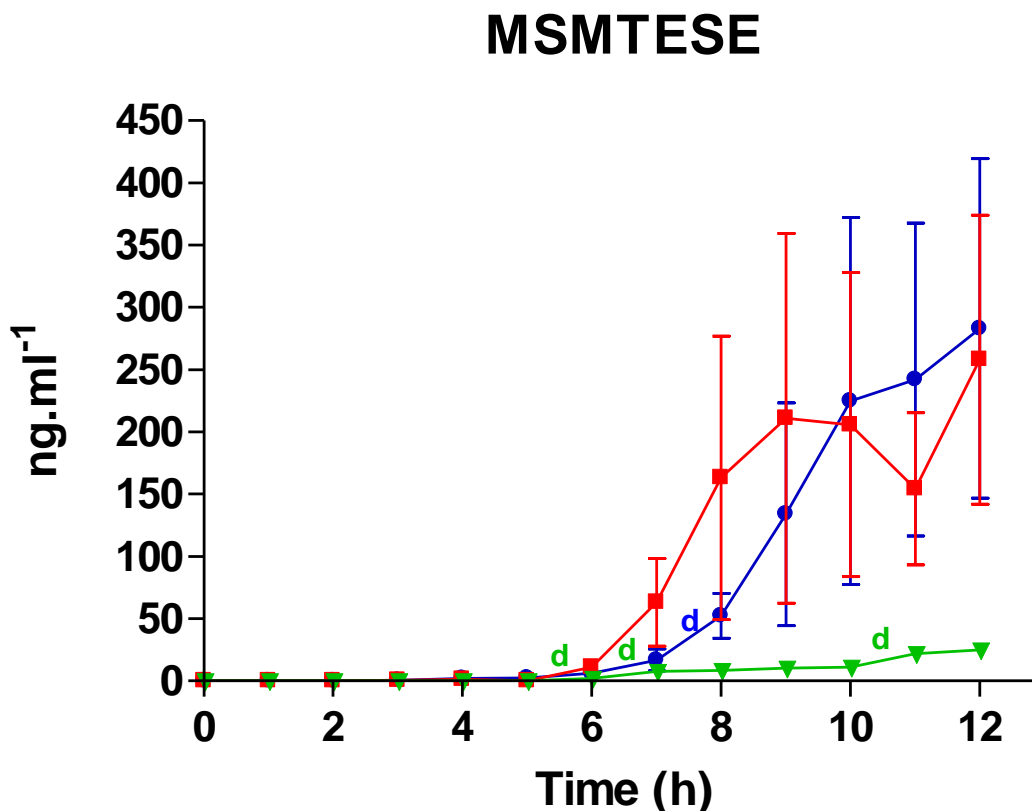


Figure 5. Changes in the HD metabolite MSMTESE concentration following exposure to $100 \mu\text{g.kg}^{-1}$ HD (▼ - 12 h tox; $n=7$ and ● - HD Controls; $n=6$), or NAC treated (■; $n=8$). Data are expressed as mean \pm SEM. 'd' represents a death in the associated group.

Thiols

Total lung tissue thiol concentration was significantly increased in the NAC treated animals when compared to HD controls (Figure 8; $p<0.05$). Total thiol levels were not significantly different within the control group or within the NAC group irrespective of lobe studied or whether the sample was taken from the left or right orientation (Figure 9).

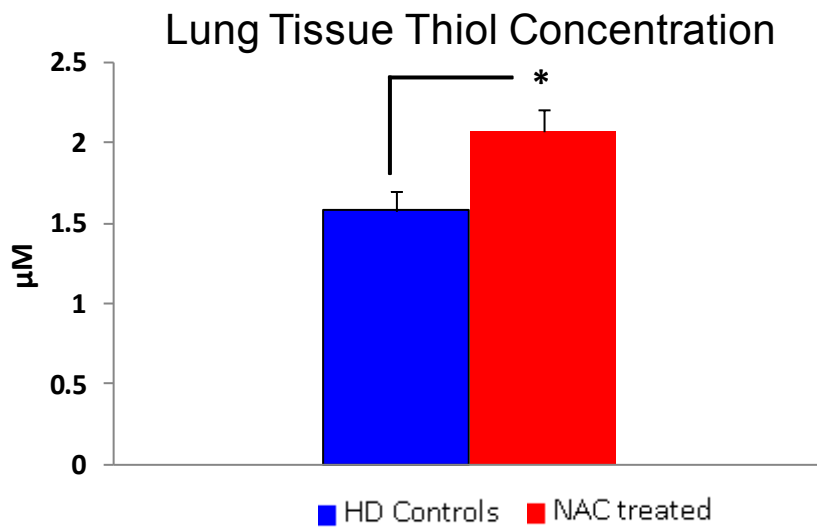


Figure 6. Concentration of total lung thiol, pooled across all lobes (left and right apical, medial and caudal). *p < 0.05 indicates significant increase in thiol concentrations in the NAC treated animals compared to HD controls.

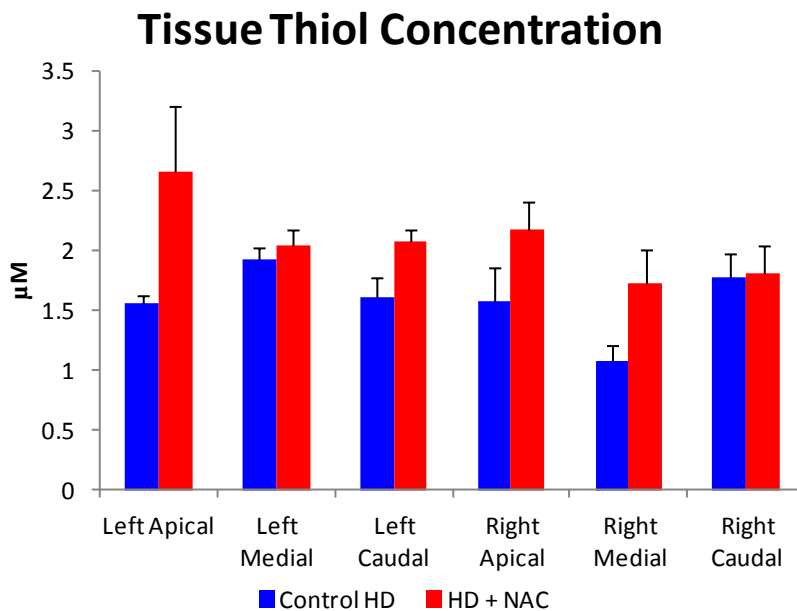


Figure 7. Concentration of lung tissue thiol in individual lobes from HD controls and NAC treated animals.

1.3.4 Markers of inflammation

Terminal BAL fluid analysis revealed a normal cellular population in the alveolar regions of air exposed controls (>85% alveolar macrophages and < 5% neutrophils) at 12 h. There were no significant differences in the numbers of alveolar macrophages, eosinophils, lymphocytes or monocytes between air and HD exposed groups from the 12 h toxicology study. There was a small increase in the number of neutrophils within the lungs of the HD exposed animals compared to air exposed animals (Figure 8).

Differential WBC count analysis of terminal BAL fluid showed an increase in the number of neutrophils recruited to the lungs of HD control animals in the 12 h treatment study (compared with air controls from the 12 h toxicology study). NAC treated animals had a significantly smaller percentage of neutrophils and concomitant higher percentage of lymphocytes in the lung than HD controls (Figure 8; * $p < 0.05$).

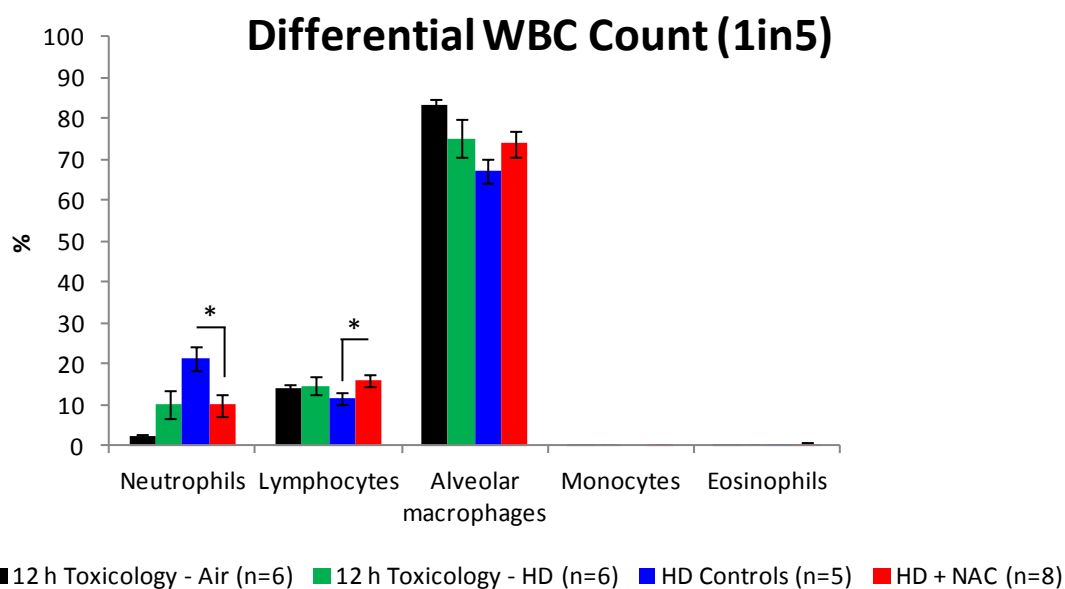


Figure 8. Terminal BAL fluid differential WBC count. (Note: $n=6$ for 12 h Toxicology HD and $n=5$ for HD controls as no lavage obtained from one animal in each group). * $p < 0.05$ indicates a significant difference in percentage of neutrophils and lymphocytes in the NAC treated group compared to HD controls.

In the 12 h toxicology study there was a small but non-significant increase in the amount of protein present in terminal BAL fluid of HD-exposed animals compared with air controls (Figure 9). In the treatment study the amount of protein in terminal BAL fluid was increased in HD control animals compared with air controls from the 12 h toxicology study. The amount of protein in the terminal lavage fluid was significantly lower in the NAC treated group than in HD control animals (Figure 9; * $p < 0.05$).

BAL fluid protein concentration

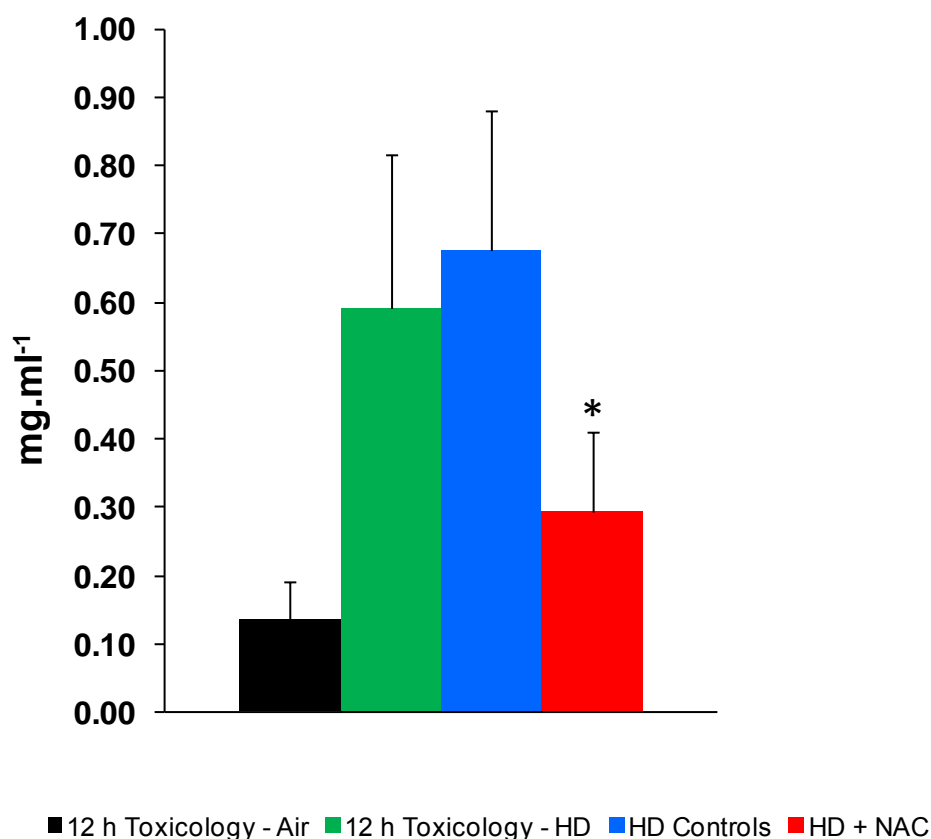


Figure 9. Terminal BAL fluid protein concentration. (Note: n=6 for 12 h Toxicology HD and n=5 for HD controls as no lavage obtained from one animal in each group). *p<0.05 indicates a significant difference in amount of protein in terminal BAL fluid of NAC treated animals compared to HD controls.

1.3.5 Pathology

Pathological assessment in the 12 h toxicology study showed intact ciliated tracheal epithelium and normal lamina propria and submucosal structures in the air control group. Areas of epithelial necrosis, septal and perivascular edema and cell debris in the lumen were identified in the HD group at 12 h, showing a progression of injury to 12 h in the HD group compared to the 6 h dose ranging study (Figures 10 and 11).

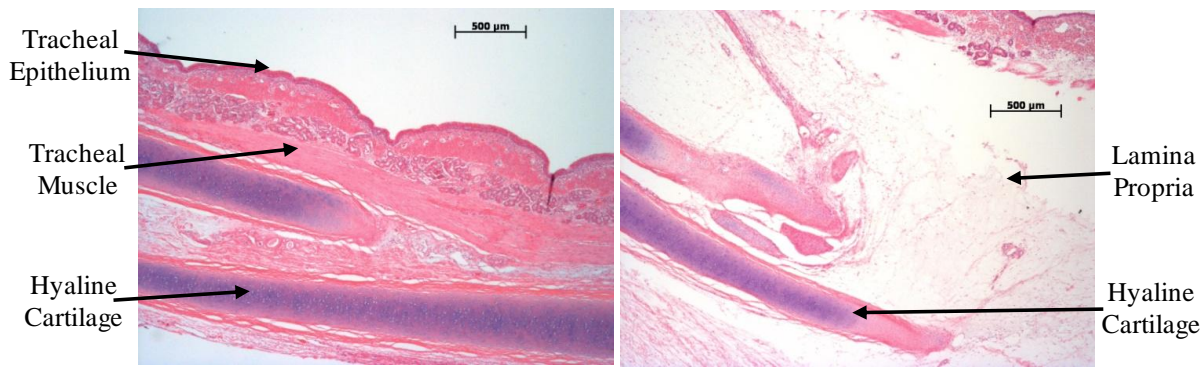


Figure 10. Transverse section through the trachea of an air control (left) and HD exposed (right) animal (x25).

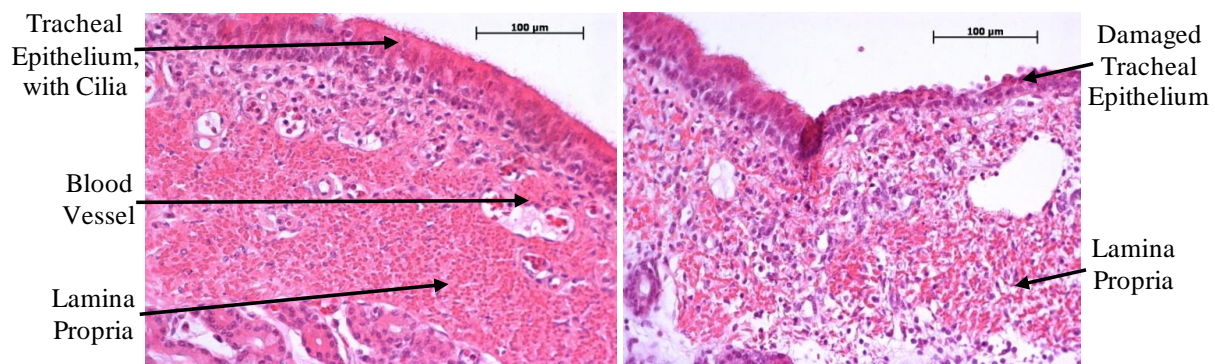


Figure 11. High power image of transverse section through the trachea of an air control (left) and HD exposed (right) animal from the 12 h toxicology study (x200).

Tracheal rings from above and below the tip of the ET tube were compared visually for both HD control and NAC treated animals (Figure 12). Tracheal rings taken from animals above the tip of the ET tube in both groups (therefore protected from the HD injury) were indistinguishable from 12 h toxicology air exposed animals' samples taken from both above and below the ET tube. Damage was therefore confined to the areas below the ET tube in the HD control and NAC treated groups. There was damage to all tracheal rings that had been exposed to mustard, with evidence of inflammation. These effects were visually less pronounced in the NAC treated animals.

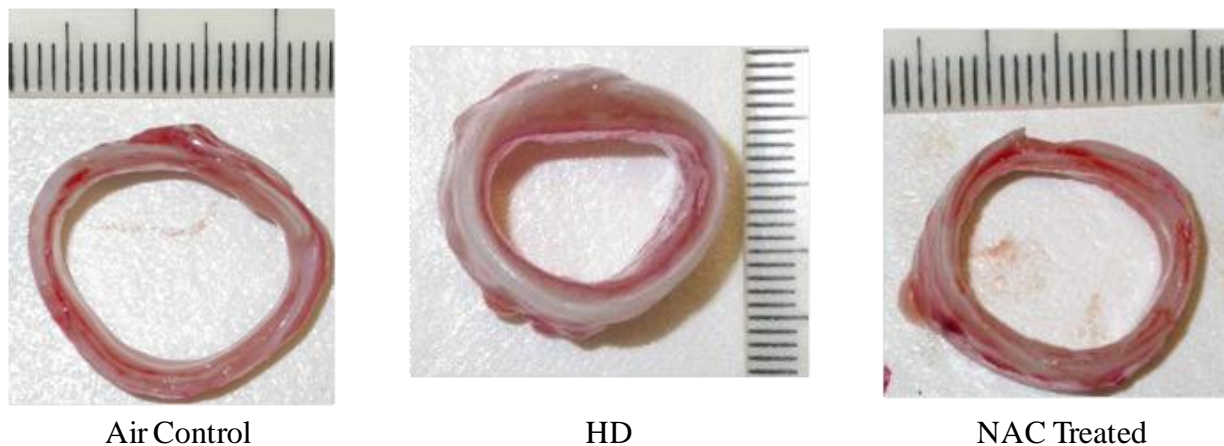


Figure 12. Tracheal rings taken from below the end of the ET tube showing clear evidence of swelling in the HD control which is absent or reduced in the NAC treated example.

In HD control animals there appeared to be extensive HD-induced damage to the bronchi compared to the NAC treated animals. The presence of excessive mucus and epithelial sloughing were also more apparent (Dstl/TR60201).

Following histopathological assessment, average scores for both HD control and NAC treated groups were significantly increased when compared to the 12 h air exposed animals (4.7 ± 0.37 ; $p < 0.05$). There was no significant difference between the NAC treated and HD controls.

Group	No. of Animals	Average Score \pm SEM
HD Controls	6	34.50 ± 1.77
NAC Treated	8	27.63 ± 1.57

Table 1. Comparison of average lung scores for HD control and NAC treated groups

1.4 Discussion

Over the course of the programme we have established a robust terminally anesthetized large animal model of HD-induced ALI in which the efficacy of potential COTS or near to market therapies for the amelioration of ALI can be investigated.

The success of this model has been, in part, due to refinements made in the measurement of HD vapor delivered to the lung with the adoption of the Gasmeter™(FTIR) analyzer. This has allowed the inhaled dose of HD to be much more tightly controlled as seen by the very small degree of variation between individual exposures, and produced a more consistent, reproducible injury. Use of the Gasmeter has also decreased reliance on offline analysis using Porapak GC-MS techniques. This has meant that studies could be conducted more intensively, producing faster results and providing better value for money.

In our model, exposure to HD vapor resulted in the development of an ALI by 12 hours post exposure. Changes in a number of physiological parameters were seen from 6 hours (decreased arterial blood oxygenation, bicarbonate, base excess and pH, with increased arterial blood pCO₂) confirming the presence of a respiratory acidosis in these animals. The shunt fraction (proportion of blood passing through the lungs which remains un-oxygenated due to perfusion and ventilation mismatch) was significantly increased compared to baseline values and the heart may be compensating for this as seen by the significantly increased heart rate at the same time point. Terminal BAL fluid analysis showed an increased number of neutrophils recruited into the lungs, and small increase in protein concentration. However, the EVLW, LWW:BW and LWW:DW ratios remained unchanged at 12 h when compared to historical air exposed animals. Accumulation of neutrophils in air spaces is consistently seen in ALI in both humans and in animal models (Pittet *et al.*, 1997) and it is postulated that they contribute either directly, or indirectly to lung injury (Wiener-Kronish *et al.*, 1990). Pathological analysis revealed damage confined to areas below the ET tube, with evidence of inflammation, epithelial sloughing and mucus production in agreement with Allon *et al.*, (2009) who reported sloughing of the tracheal epithelium, necrosis, edema and blood congestion in the lamina propria in their guinea pig model of inhaled HD-induced ALI. MSMTESE could be detected in urine from 4 hours post HD exposure. MSMTESE, unlike thiodiglycol and its sulfoxide derivative (Balali-Mood and Hefazi, 2005), is an unequivocal marker of HD exposure which can be reduced to thioether derivatives for analysis by GC-MS (Black and Read, 1988). Urinary MSMTESE could provide a valuable diagnostic tool in identifying casualties exposed to inhaled HD prior to clinical signs. Concentrations of MSMTESE were much higher in the treatment study (for both HD controls and NAC treated animals) when compared to animals exposed to HD in the previous 12 h toxicology study. Calibration data was compared with that from the previous experiments and no reason was found for the high levels being due to analytical error, with the calibration data consistent, though variations in the instrument sensitivity were apparent. We are, therefore, at this time unable to explain the apparent difference between the two studies.

Post exposure treatment with multiple inhaled doses of nebulized NAC was effectively able to reverse or reduce a number of the physiological effects of HD exposure. These include significantly improved arterial blood oxygenation, reduced heart rate and shunt fraction, as well as other markers of lung injury. We have shown a significantly reduced number of neutrophils and protein concentration in the terminal BAL fluid of NAC treated animals, as well as a significantly increased lung tissue thiol concentration compared with HD controls. However, we are unable to state whether inhaled HD reduced tissue thiol concentrations compared with air

exposed control animals. Pathological effects of HD vapor were visually less pronounced in the NAC treated animals, where there appears to be a subjective decrease in the severity of the HD injury observed at *post mortem*.

NAC is known to have mucolytic and anti-oxidant properties and, as a synthetic derivative of cysteine, has a role as a glutathione donor. HD exposure is associated with inflammatory and oxidative injury causing damage in both the upper and lower respiratory tract. It has been postulated to deplete reduced GSH levels (Anderson *et al.*, 2009). Glutathione depletion and oxidative stress are key events contributing to cell death. Decreased cell matrix adhesion, increased mucus production and loss of ciliary function have all been reported following HD exposure *in vitro* (Pohl *et al.*, 2009). It has not been possible to fully elucidate the beneficial mechanism of action of NAC in this study; however, our results show a significant reduction in neutrophil infiltration into the lungs, indicating an anti-inflammatory effect of NAC. NAC has been reported to down regulate the activity of the AP-1 transcription factor, contributing to reduced infiltration of inflammatory cells into alveolar spaces, although this study used the half mustard analogue CEES, rather than HD (Mukhopadhyay S *et al.*, 2009). The ability of NAC to protect against HD-induced lung injury could also be explained by both its ability to raise intracellular concentrations of cysteine (as shown in our study), and hence of GSH, and/or by scavenging oxidant species (Aruoma *et al.*, 1989). NAC is widely used as a mucolytic drug, reducing viscosity and elasticity of mucus, due to its ability to reduce disulphide bonds (Gillissen *et al.*, 1998). NAC has also been shown to ameliorate the symptoms of chronic lung injury, including cough and dyspnoea, in human survivors many years after HD exposure, an effect likely related to its combined anti-inflammatory and anti-oxidant activities (Ghanei M *et al.*, 2008).

Whatever the mechanism, NAC has been beneficial in this study in improving a number of key clinically relevant physiological indices affected by exposure to HD and should be further investigated as a potential COTS treatment for HD-induced ALI. The dosing protocol for this study was taken from human dosing protocols in discussion with medical colleagues. It is not possible to state whether optimization of this protocol may provide a more effective treatment strategy for this injury; however, further studies should investigate the potential effectiveness of a multi pharmacological approach, targeting a number of cellular responses, in this model.

Our model of HD-induced ALI could now also be used to test a variety of promising candidate therapies that have been shown to have benefit in small animal models of HD-induced ALI e.g. other antioxidants, protease inhibitors (Anderson *et al.*, 2009), corticosteroids and vitamin E (Wigenstam *et al.*, 2009), chemical scavengers and PARP inhibitors (Smith *et al.*, 2009). Our model would provide a second species in which to demonstrate efficacy, in a model closer to man and more representative of human exposure.

Key Research Accomplishments

- Utilizing sham exposed animals a unique and novel anesthetized spontaneously breathing porcine model that could be taken to 12 h has been established. This model has been shown to be stable and robust with the degree of injury following HD exposure highly reproducible.
- Successfully and reproducibly exposed animals to HD at the required inhaled dose with minimal variability in delivered dose.
- Assessment by lung pathology suggests this model is more representative of human HD-induced lung injury than small rodent models.
- Successfully incorporated FTIR technology using Gasmet™ therefore allowing multiple experiments to be carried out in a shorter time frame, allowing experiments to be carried out in a more cost effective manner and decreasing reliance on offline analysis.
- Identification of the HD metabolite, MSMTESE in urine as early as 4 h post exposure, and prior to physiological signs and symptoms, could lead to the development of an early diagnostic indicator of poisoning.
- Treatment with multiple inhaled doses of nebulized NAC post HD exposure was found to be effective in reversing or reducing a number of the physiological effects of HD exposure. NAC should be further investigated as a potential COTS treatment for HD-induced ALI.

Reportable Outcomes

Manuscripts

- Fairhall SJ, Brown RFR, Jugg BJA, Smith AJ, Mann TM, Jenner J and Sciuto AM. Preliminary studies of sulfur mustard-induced lung injury in the terminally anaesthetized pig: Exposure system and methodology. *Toxicology Mechanisms and Methods* 2008;18: 355-362.
- Fairhall SJ, Jugg BJA, Read RW, Stubbs SJ, Rutter SJ, Smith AJ, Mann TM, Jenner J and Sciuto AM. Exposure-response effects of inhaled sulfur mustard in a large porcine model: a 6-h study. *Inhalation Toxicology* 2010; 22(14): 1135–1143.
- Jugg BJA, Fairhall SJ, Smith AJ, Rutter SJ, Mann T, Perrott R, Jenner J and Sciuto AM. N-acetyl-L-cysteine (NAC) Protects Against Inhaled Sulfur Mustard (HD) Poisoning in the Large Swine. *Clinical Toxicology*, 2012; in preparation.

Abstracts

- Jugg BJ, Brown R, Smith AJ, Mann T, Scawin J, Masey C, Jenner J and Sciuto AM. The acute toxicological effects of inhaled sulfur mustard – studies in a porcine model. *Biosciences* 2008.
- Jugg BJ, Smith AJ, Fairhall SJ, Mann T, Jenner J and Rice P. The past and future of a porcine model of chemical induced lung injury. *Biosciences* 2010.
- Jugg BJ, Fairhall SJ, Smith AJ, Rutter SJ, Mann T, Stubbs SJ, Read RW, Jenner J and Sciuto AM. The acute toxicological effects of inhaled sulfur mustard. *CBD S&T* 2010.
- BJA Jugg, SJ Fairhall, AJ Smith, SJ Rutter, TM Mann, R Perrott, J Jenner, AC Green and AM Sciuto. Medical countermeasures for inhaled sulfur mustard. *CBD S&T* 2011.

Presentations

- Brown RFR. Preliminary studies of the effects of inhaled sulphur mustard in the terminally anaesthetised pig. *Bioscience* 2006.
- Jugg BJ. Inhalation toxicology of sulphur mustard: Dose ranging study in a porcine model. NATO research and technology agency 2007.
- Jugg BJ. The acute toxicological effects of inhaled sulfur mustard – Studies in a porcine model. *Biosciences* 2008.
- Fairhall SJ. The Acute Toxicological Effects of Inhaled Sulfur Mustard. *CBD S&T* 2010

Posters

- Scawin J, Fairhall S, Brown R, Jugg B, Smith A, Mann T, Jenner J and Sciuto AM. Preliminary studies of sulphur mustard induced lung injury in the terminally anaesthetised pig: Exposure system and Methodology. *Bioscience review*, 2006, Medical Defence Review, Baltimore, USA.
- Brown R, Jugg BJ, Smith AJ, Mann T, Fairhall SJ, Platt J and Sciuto AM. Inhalation toxicology of sulphur mustard – Dose ranging study in a porcine model. *ATS* 2007.
- Jugg BJ, Smith AJ, Mann T, Fairhall SJ, Masey C, Perrott R, Jenner J and Sciuto AM. The acute toxicological effects of inhaled sulphur mustard. *CBD S&T* 2009.
- Jugg BJ, Fairhall SJ, Smith AJ, Rutter SJ, Mann T, Stubbs SJ, Read RW, Jenner J and Sciuto AM. The Acute Toxicological Effects of Inhaled Sulfur Mustard. *CBD S&T* 2010.
- Jugg BJ, Fairhall SJ, Smith AJ, Rutter SJ, Mann TM, Perrott R, Jenner J, Green AC and Sciuto AM. Medical Countermeasures for Inhaled Sulfur Mustard. *CBD S&T* 2011.

Material Transfer Agreements (MTAs)

University of Portsmouth

Materials Transfer and Non-Disclosure Agreement, Dstl/IPR/W235, 26 November 2009.

First Amendment to Materials Transfer and Non-Disclosure Agreement Dstl/IPR/W235, March 2011.

University of Southampton

Materials Transfer & Non-Disclosure Agreement 20110411-W311 with the University of Southampton, April 2011.

University of Newcastle

Materials Transfer Agreement 20110725-W335 MTA with the University of Newcastle, July 2011.

Conclusions and Recommendations

- A novel model to investigate the acute toxicology of inhaled sulfur mustard has been established.
- This model is representative of human exposures and suitable for the assessment of therapeutic interventions.
- NAC has been shown to be effective in reversing or reducing a number of the physiological effects of HD exposure.
- NAC, as a COTS product, could be rapidly transitioned to military and civilian treatment protocols for inhaled HD poisoning.
- Further assessment of NAC is recommended to optimize the treatment protocol and assess effectiveness over a longer timeframe.
- Other promising treatment candidates identified from small animal experiments should be transitioned into the larger animal model to comply with the two species rule for drug licensing.
- Continued research will further enhance evidence based treatment protocols for HD induced lung injury.

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Appendix

The Toxicity of Inhaled Sulphur Mustard in a Large Animal Model

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Dstl/TR60201

March 2012

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Executive summary

Exposure of military and civilian populations to inhaled toxic chemicals can take place as a result of deliberate release (warfare, terrorism) or following accidental releases from industrial concerns or transported chemicals. Sulphur mustard (HD) is best known as a blister agent but the majority of deaths are associated with pulmonary damage. Inhalation of HD vapour can cause life threatening lung injury for which there is currently no specific medical therapy, available treatment remaining largely supportive. This treatment often requires intensive care facilities which may become overwhelmed in mass casualty events, and may be of limited benefit in severe cases [1]. There, therefore, remains a need for evidence based treatment strategies to inform both military and civilian medical response teams on the most appropriate treatment for chemically induced lung injury. A reproducible, characterised *in vivo* model is required to produce this evidence base for novel therapies targeting HD-induced lung injury.

N-acetyl-L-cysteine (Mucomyst™; NAC) is a thiol compound with mucolytic and anti-oxidant actions and is an important pre-cursor of cellular glutathione synthesis. It has been shown in previous studies to have some benefit as a treatment for HD-induced lung injury [2,3].

The aim of this programme was to determine the efficacy of nebulised NAC as a post exposure treatment for the respiratory effects of HD vapour exposure in a clinically relevant, reproducible large animal model of HD exposure [4,5,6]. This report covers all of the data recorded from the NAC treatment study, and compliments the peer reviewed publication Jugg *et al.*, 2012 [7].

Anaesthetised, spontaneously breathing large white pigs were randomly assigned to either HD control (100 µg.kg⁻¹ HD; n=6) or HD treated (100 µg.kg⁻¹ HD followed by NAC (1 ml of 200 mg.ml⁻¹ solution of Mucomyst™ at + 30 min, 2, 4, 6, 8 and 10 hours post exposure; estimated total dose delivered 1200 mg; n=8)) groups. Cardiovascular and respiratory parameters were recorded continuously throughout the experiment. Blood and bronchoalveolar lavage (BAL) fluid were collected to allow blood gas and haematology analysis, and to assay for lung inflammatory cells and mediators. Urine was collected and analysed for HD metabolites. Tissue samples were taken *post mortem* and processed for histopathological analysis and tissue thiol concentrations.

In the HD exposed group 5 /6 animals survived to 12 hours. Arterial blood oxygenation (PaO₂) and oxyhaemoglobin levels were significantly decreased at 12 hours, with animals becoming hypoxemic. Shunt fraction was significantly increased at 12 hours. Respiratory acidosis was apparent with arterial blood carbon dioxide (PaCO₂) increased and arterial blood pH and bicarbonate (HCO₃⁻) significantly decreased at 12 hours. A urinary adduct (1-methylsulfinyl-2-[2(methylthio)ethylsulfonyl]ethane] (MSMTESE) was detected 4 hours post exposure with levels increasing until the end of the 12 hour study. Pathological examination revealed areas of oedema, inflammatory cell infiltration and necrosis of the tracheal epithelium. These findings are consistent with those seen in the early stages of acute lung injury (ALI).

In the NAC treated group all animals survived to 12 hours (n=8). There was significantly improved arterial PaO₂, HCO₃⁻ levels and shunt fraction compared to HD controls. There were significantly fewer neutrophils and lower concentrations of protein in BAL fluid compared to HD controls. NAC's mucolytic and anti-oxidant

properties may be responsible for the beneficial effects seen: improving clinically relevant physiological indices affected by HD exposure. Further therapeutic benefit may result from optimisation of the treatment protocol or from a combination therapy approach.

This is the first time that a treatment has been shown to be efficacious for HD-induced lung injury in two animal species [3]. As NAC is a COTS drug it could be rapidly transitioned into front line medical care.

Any opinions, findings and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the USAMRAA or USAMRICD.

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1 Introduction

The use of sulphur mustard (bis(2-chloroethyl)sulphide; HD) as a chemical warfare (CW) agent remains of concern to the Armed Forces. Since its first use in World War 1 [8] HD has been used in numerous locations across the world by aggressors [9]. This includes during the Iran-Iraq conflict in the 1980s where the use of HD resulted in over 100,000 medical casualties [10]. Though primarily a dermal vesicant, it is the inhaled effects of HD which account for the majority of deaths following HD poisoning. Indeed, the majority of deaths from HD poisoning during WW1 had some degree of pulmonary involvement [8]. The respiratory effects following exposure to HD vapour range from an unproductive cough, bronchitis, necrosis of the respiratory epithelium, bronchopneumonia, pulmonary oedema to respiratory failure; often a consequence of complications such as infection [11,12].

Currently no pharmacologic therapy exists for the treatment of HD-induced acute lung injury (ALI), with treatment being supportive (ventilation and supplementary oxygenation) provided in an intensive care environment. This places a high burden on civilian/military medical staff and healthcare systems could be easily overwhelmed in a mass casualty event. Hence, there is an ongoing requirement to find efficacious treatments for chemically-induced ALI.

Previous work in this laboratory has established a clinically relevant, reproducible large white pig model of HD-induced ALI, in which to assess the efficacy of therapeutic candidates [5]. The use of the pig allows the upper respiratory tract (URT) to be bypassed, enabling the effects of HD when delivered directly to the lung to be investigated [4]. A 6 h dose ranging study identified the doses of HD that produced a range of injuries, from which a further assessment of the toxicological profile of HD exposure [13] identified changes (mild to moderate epithelial necrosis, multifocal areas of inflammation with inflammatory infiltrates (mainly neutrophils)) consistent with clinical descriptions from human poisoning, allowing confidence to assess potential treatment strategies for HD-induced ALI [11,12].

Previous studies have shown some benefit in the use of NAC as a treatment for HD-induced lung injury [2,3]. Anderson *et al* [3] provided evidence of NAC-induced protection against HD-induced lung injury in rats given NAC (816 mg.kg^{-1} , i.p.) at the start of exposure to HD vapour (reduced total protein concentration and neutrophil number in bronchoalveolar lavage fluid at 24 hours post exposure).

NAC has a number of potential therapeutic effects for HD-induced ALI; it is a thiol (SH) containing compound with mucolytic properties (cleaving the sulphydryl bonds in pulmonary mucus) used to reduce viscosity and elasticity of mucus [14]. The SH group exerts direct anti-oxidant actions enabling NAC to scavenge hydrogen peroxide (H_2O_2), hydroxyl radicals and hypochlorous acid [15]. The glutathione (GSH) redox cycle removes H_2O_2 by dismutation into H_2O and O_2 . NAC can also be de-acetylated to cysteine (an important pre-cursor of cellular GSH synthesis) thereby stimulating the cellular GSH system [16].

The aim of the study reported here was to determine the efficacy of nebulised NAC as a post exposure treatment for the respiratory effects of HD vapour exposure in a model more representative of human exposure.

2 Methods

2.1 Animals and surgical preparation

All animal work was carried out in accordance with the UK Animals (Scientific Procedures) Act 1986, and was approved by Ethical Review at Dstl, Porton Down, UK.

Large white female pigs (49-59 kg, mean weight 52.8 ± 2.6 kg) ($n = 16$) were obtained from a commercial source. The animals were housed individually in straw lined pens, and were allowed food (standard pig diet) and water *ad libitum* for 5 days prior to the experiment. From 12 h prior to the experiment access to food was withdrawn though water remained freely available.

2.1.1 Surgical procedure

Animals were premedicated with midazolam hydrochloride ("Hypnovel", Roche Products Ltd, UK) by intramuscular injection (0.6 mg.kg^{-1}), and anaesthesia induced by inhalation of 5 % isoflurane ("IsoFlo", Abbott Laboratories, UK) in 70 % oxygen and 30 % nitrous oxide. The animals were intubated with a size 10 oral endotracheal (ET) tube which had been lined with polytetrafluoroethylene (PTFE) as previously described [4]. Where intubation was not viable due to the inflexible nature of the lined tube (and due to unusually small airway diameters), the ET tube was placed by tracheostomy (following approval by the UK HO Inspector and discussion with Dr Sciuto (USAMRICD)). Nine of the 14 animals included in the study underwent the tracheostomy procedure. Surgery was performed under aseptic conditions by cut down procedure for insertion of arterial and venous catheters. A 'Pulse Contour Continuous Cardiac Output' (PiCCO) catheter was inserted into the femoral artery and attached to a PiCCO monitor, allowing continuous measurement of cardiac output, systemic artery pressure, stroke volume, and core temperature. Periodic thermal dilution analysis allowed estimation of extravascular lung water (EVLW). Other non invasive physiological monitoring devices were attached to a Propaq 106EL monitor. In the pig the presence of a sub-urethral diverticulum makes urethral catheterisation difficult and, therefore, a Bonanno catheter was introduced into the bladder via an open cystotomy. Throughout the study a maintenance infusion of 0.9% w/v sodium chloride and 4% w/v glucose ($2.5 \text{ ml.kg}^{-1}.\text{h}^{-1}$) was delivered to replace insensible losses. Once surgery was completed intravenous anaesthesia was established and maintained using propofol 1% ($10 - 12 \text{ mg.kg}^{-1}.\text{h}^{-1}$) (Fresenius Propoven®, Fresenius Kabi Ltd, Cheshire, UK) and alfentanil hydrochloride ($0.5 - 2.5 \text{ µg.kg}^{-1}.\text{h}^{-1}$) (Rapifen® Janssen-Cilag Ltd, McGregor Cory Ltd, UK).

2.1.2 Exposure to HD

The system for exposing animals to HD has been described in detail [4]. In brief, the animals were maintained supine and allowed to breathe spontaneously while baseline measurements were taken for 1 h. They were then connected to the exposure apparatus via a PTFE-lined ET tube, a size 2 Fleisch pneumotachograph and a sample port for 10 min.

HD concentration was measured in real time using a Gasmet™ Fourier Transform Infrared (FTIR) spectrometer (Quantitech, UK). Prior to this study the Gasmet was characterised and evaluated (Annex A). To confirm the Gasmet results, off-line analysis was run in parallel using Porapak absorption tubes. These were analysed by solvent (hexane) elution and quantitative gas chromatography-flame photometric

detection (GC-FPD). For this a mid-polar capillary column was used and the mobile phase was helium carrier gas at a flow rate of 2.5 ml.min⁻¹. The retrospective analysis was carried out in a UKAS accredited laboratory.

For the exposures a specific inhaled dose of 100 µg.kg⁻¹ was targeted. This was calculated using concentration, time, the animal's body weight and total inhaled volume ^{*1}. Effort was made to maintain the animals' minute volumes between 6 and 8 l.min⁻¹ by controlling the dose of anaesthetic. The inhaled dose was then estimated every minute during the exposure, and the exposure terminated when the inhaled dose reached the desired value. Thus, whilst the exposure duration was planned to be 10 min, in practice it varied between 12 and 19 min to account for uncontrollable variations in the animal's minute volume.

2.1.3 Groups

16 animals were entered into the study. One animal died prior to exposure following unsuccessful intubation under anaesthesia. A further animal was excluded from the study following statistical analysis which showed it to be an outlier having only a very mild injury at 12 h.

Animals were randomly assigned to either HD control (100 µg.kg⁻¹ HD; n = 6) or HD + NAC treated (100 µg.kg⁻¹ HD followed by NAC; 1 ml of 200 mg.ml⁻¹ solution of Mucomyst™ at + 30 min, 2, 4, 6, 8 and 10 h post exposure; estimated total dose delivered approached 1200 mg; n=8) groups.

2.1.4 Treatment

NAC was administered as an aerosol generated by an Aeroneb® Lab micropump nebuliser (Aerogen (Ireland) Ltd, Galway Ireland). The nebuliser was placed at the end of the ET tube. A "spacer" (approx. 25 cm plastic tube) was attached to the other end of the nebuliser to reduce the amount of drug lost during expiration. The nebuliser was run until dry (approx. 5 min). Control animals had the nebuliser and spacer attached for 5 min (to ensure any effects of increased dead space were replicated), but the nebuliser was not switched on.

2.2 Physiological measurements

Cardiac and pulmonary physiological parameters were recorded every 5 min for the first 30 min following exposure and then every 30 min throughout the course of the experiment (up to 12 h). Derived variables were calculated using published formulae [17].

Arterial and mixed venous blood samples were taken prior to exposure, immediately post exposure, at 30 min post exposure and then hourly post exposure for the duration of the experiment. At each time point aliquots of arterial and mixed venous blood were placed into a heparinised blood gas syringe (Sims Portex Ltd, UK) and immediately analysed using a GEM Premier 3500 blood-gas analyser (Instrumentation Laboratories UK Ltd, Cheshire, UK). A second aliquot of arterial blood was taken into EDTA, mixed and refrigerated (4 °C) for later haematological analysis. A third sample was separated into appropriate anticoagulants, centrifuged

^{*1} Inhaled Dose (mg.kg⁻¹) = $\frac{\text{Concentration (mg.m}^{-3}) \times \text{Inhaled Volume (m}^3\text{)}}{\text{Body Weight (kg)}}$

(3000 rpm, 10 min) and the plasma stored (-80 °C) for analysis. In addition to the collection of blood, total urine output was recorded hourly and aliquot samples retained (-80 °C).

Respiratory parameters were recorded in real time using eDAcq (EMMS Data Acquisition) software (Infodisp, UK). Minute and tidal volume, lung resistance and compliance, together with other lung function variables, were monitored in real time throughout, and subsequent to, the exposure.

2.2.1 Suctioning

An hourly suctioning protocol was started at 6 h post exposure in order to remove any obstructions from the ET tube. To ensure that the suction tube did not go beyond the end of the ET tube the suction line was cut to size, preventing the removal of any debris or secretions from the lungs. If signs of severe respiratory distress (a large drop in arterial pO₂ over a period of less than 5 min) were exhibited prior to 6 h, then the ET tube was suctioned to clear any obstructions.

2.3 Post mortem and histopathology

At the end of the 12 h monitoring period, or when the animal became moribund (defined as asystole and a mixed venous oxygenation (SvO₂) of less than 15%) the animal was culled by an intravenous overdose (20 ml) of sodium pentobarbitone, 200 mg.ml⁻¹ ("Euthatal" Rhone Merieux Ltd., Harlow, Essex) and a full *post mortem* (PM) examination carried out. PM was carried out wearing personal S10 respirators to prevent exposure from any potential HD 'off-gassing'. Following removal of the ET tube the trachea was clamped, the thorax opened and the lungs and heart were removed. The lungs were removed to a fume cupboard and a small incision made in the trachea just below the clamp. A flexible bronchoscope (Fiberoptic Bronchoscope, Olympus BF-4B2) was then inserted and manoeuvred into the right median lobe. Aliquots of sterile saline (4 x 40 ml) were expelled into the lobe, the fluid aspirated between each aliquot and the samples pooled and placed on ice. Following lavage, the heart and any excess tissue were removed and the lungs were then weighed for lung wet weight to body weight (LWW:BW) ratio determinations (the weight of the un-recovered lavage saline being taken into account).

Lung samples (approx. 3 g of tissue) were taken from each of the lobes, weighed and then dried in an oven (40 °C). The samples were weighed daily until a stable weight had been achieved (approx. 5 days), to determine lung wet weight to dry weight (LWW:DW) ratios.

Lung samples (approx. 1 g of tissue) were taken from each lobe for genomic analysis, stabilised in RNAlater solution (10 ml per g of tissue) and stored overnight (4 °C) to allow the fluid to completely penetrate the tissue, before being transferred to -80 °C storage until shipment to the US Army Medical Research Institute of Chemical Defense (USAMRICD) for analysis.

Lung samples (approx. 1 g of tissue) were also taken to determine tissue protein and non-protein thiol concentrations (also called sulphydryls). The tissues were weighed and then homogenised in Dulbecco's phosphate buffer (5 ml per g of tissue). The homogenate was centrifuged (1800 rpm for 10 min), the supernatant removed and stored (-80 °C) for later analysis.

Samples from each of the lobes, together with samples from other major organs were taken, fixed in neutral buffered formalin and processed for histopathological examination using routine methods.

2.3.1 Sample Processing

Histology slides were sent to the Veterinary Laboratories Agency (VLA) for a qualified independent pathologist to score the slides for pathological changes (Annex B).

A bespoke scoring system was developed to assess lung pathology, based on:

- Bronchiolar epithelial degeneration and necrosis
- Hypertrophy/hyperplasia of bronchiolar epithelium
- Cell debris in the bronchiolar lumen
- Perivascular (and peribronchiolar) oedema, fibrin and cell infiltrates
- Alveolar oedema and fibrin
- Alveolar cell infiltrates
- Interstitial (septal) oedema
- Haemorrhage

These parameters were assessed against the criteria shown in Table 1. The samples were read blind and the lung lobe scores were summed for each animal.

Severity	Score
Not Observed	0
Mild	1
Moderate	2
Severe	3

Table 1. Severity criteria against which histopathological parameters were scored.

2.3.2 Thiols

Thiols were quantitated using the Molecular Probes Thiol and Sulfide Quantitation Kit (Life Technologies Ltd., Paisley, UK) (Annex C). Thiols reduce disulfide-inhibited papain to release active enzyme. Papain activity is measured using its reaction with N-benzoyl-L-arginine p-nitroanilide to release p-nitroaniline. The absorbance of p-nitroaniline is measured at a wavelength of 412 nm. Calibrations were performed against L-cysteine and the linear range is approximately 0.2 to 1.5 μ M. Samples with thiol concentrations exceeding 1.5 μ M were diluted prior to re-analysis.

2.3.3 Haematology

A standard 8 test haematological analysis was carried out on arterial blood samples and BAL fluid (4 °C) using a Siemens Advia 120 installed with 2120 software. Differential white blood cell (WBC) counts (macrophages, lymphocytes, monocytes, eosinophils, neutrophils and basophils) were obtained manually from blood smears and BAL fluid slides (Shandon Cytospin, 1800 rpm, 10 min) stained with DifQuik, using a Zeiss Axioskop 40 microscope. The remaining BAL fluid was centrifuged (10 min, 3000 rpm) and the supernatant used to determine protein content (Coomassie blue). Supernatant was stored in 20 ml aliquots (-80 °C) for analysis of inflammatory mediators (IL-1 β , IL-6, IL-8 and TNF α) using commercially available standard quantitative porcine ELISA kits (R&D Systems, Abingdon, UK).

2.3.4 Markers of neutrophil activation

Analysis of desmosine in urine and terminal BAL fluid, and matrix metalloproteinase (MMP), neutrophil elastase (NE) and myeloperoxidase (MPO) activity in terminal BAL fluid was carried out at the University of Portsmouth, UK, using commercially available reagents (Annex D).

2.3.5 Urine adducts

Urine aliquots were analysed for the mono-sulfoxide β -lyase metabolite (1-methylsulfinyl-2-[2(methylthio)ethylsulfonyl]ethane], MSMTESE) using the method published by Read and Black [18], modified by use of an alternative C18 liquid chromatography (LC) column (ACE 3 C18HL, 150 mm x 2.1 mm; Hichrom Ltd, Reading, UK). Calibration was performed using urine (from pigs exposed to air in the dose ranging study) that had been spiked with MSMTESE over the range 0.1 to 1000 ng.ml⁻¹. Deuterated (D6) MSMTESE was used as internal standard for quantitation.

2.4 Statistical Analysis

All data are expressed as mean \pm SEM. Differences between the HD control and NAC treated groups were investigated at 3, 6, 9, and 12 h post exposure. The average data values between these two groups at individual time points were compared using a two-sample t-test. Assessment of the distribution of the data was performed in order to justify the use of the two-sample t-test. If there was evidence against the data following a normal distribution, a non-parametric alternative (Wilcoxon) was used. Tissue thiol concentrations were analysed using a three way ANOVA with repeated measures (treatment effect; section effect (left or right lung); or lobe effect (apical, medial or caudal)).

3 Results

3.1 Survival

In the HD control group five animals survived to the end of the study with one animal dying 7.5 h after exposure from HD-induced ALI. All NAC treated animals survived to 12 h post exposure.

3.2 HD Exposures

Table 2 show's that the target and achieved inhaled doses for both groups were not significantly different. Achieved mean inhaled dose = $99.8 \pm 1.1 \mu\text{g.kg}^{-1}$. The results confirm the accuracy and reproducibility of the exposure system and highlight superiority of targeting inhaled dose over concentration time profile (Ct).

Group	Target Inhaled Dose ($\mu\text{g.kg}^{-1}$)	Average Inhaled Dose \pm Standard Error ($\mu\text{g.kg}^{-1}$)
HD Control	100	101.85 ± 1.66
NAC Treated	100	98.23 ± 1.20

Table 2. Target inhaled dose verses achieved inhaled doses for both groups.

3.3 Physiology

3.3.1 Oxygenation

Arterial blood oxygenation (PaO_2), saturation and oxyhaemoglobin levels were significantly decreased in the HD control animals at 12 h post exposure ($\dagger\dagger\dagger p < 0.001$) compared to baseline values, with a concomitant increase in reduced haemoglobin (RHb) levels (Figures 1 and 2). The NAC treated group maintained arterial blood oxygenation at baseline levels throughout. The NAC treated group had significantly higher PaO_2 , saturation, oxyhaemoglobin and lower RHb at 12 h ($*p < 0.05$) compared to HD controls.

Total haemoglobin (THb) levels in the HD control animals were significantly lower than in the NAC treated animals at 3 h post exposure ($*p < 0.05$). THb remained stable in the NAC treated animals throughout the 12 h study. Levels of methaemoglobin were also significantly reduced in the HD control animals when compared to NAC treated animals at 12 h ($*p < 0.05$) (Figure 2).

3.3.2 Arterial blood physiology

Arterial blood pH was significantly reduced in the HD control ($\dagger\dagger p < 0.01$) and increased for NAC treated animals at 12 h compared to baseline values ($\wedge p < 0.01$). However, there was no significant difference between the HD control and NAC treated groups over the course of the study (Figure 1).

Bicarbonate concentration was significantly decreased in HD controls at 12 h when compared to baseline values, suggesting a respiratory acidosis in these animals ($\dagger p < 0.05$) which was reflected in the base excess ($\dagger p < 0.05$). Bicarbonate concentration was significantly lower in the HD control animals compared to the NAC treated animals at 12 h ($*p < 0.05$). NAC treated animals maintained normal bicarbonate levels, and hence base excess, throughout the study. HD control animals

had significantly reduced arterial blood oxygenation at 12 h when compared to the NAC treated animals (* $p < 0.05$) with a concomitant significant increase in arterial blood carbon dioxide concentration (* $p < 0.05$).

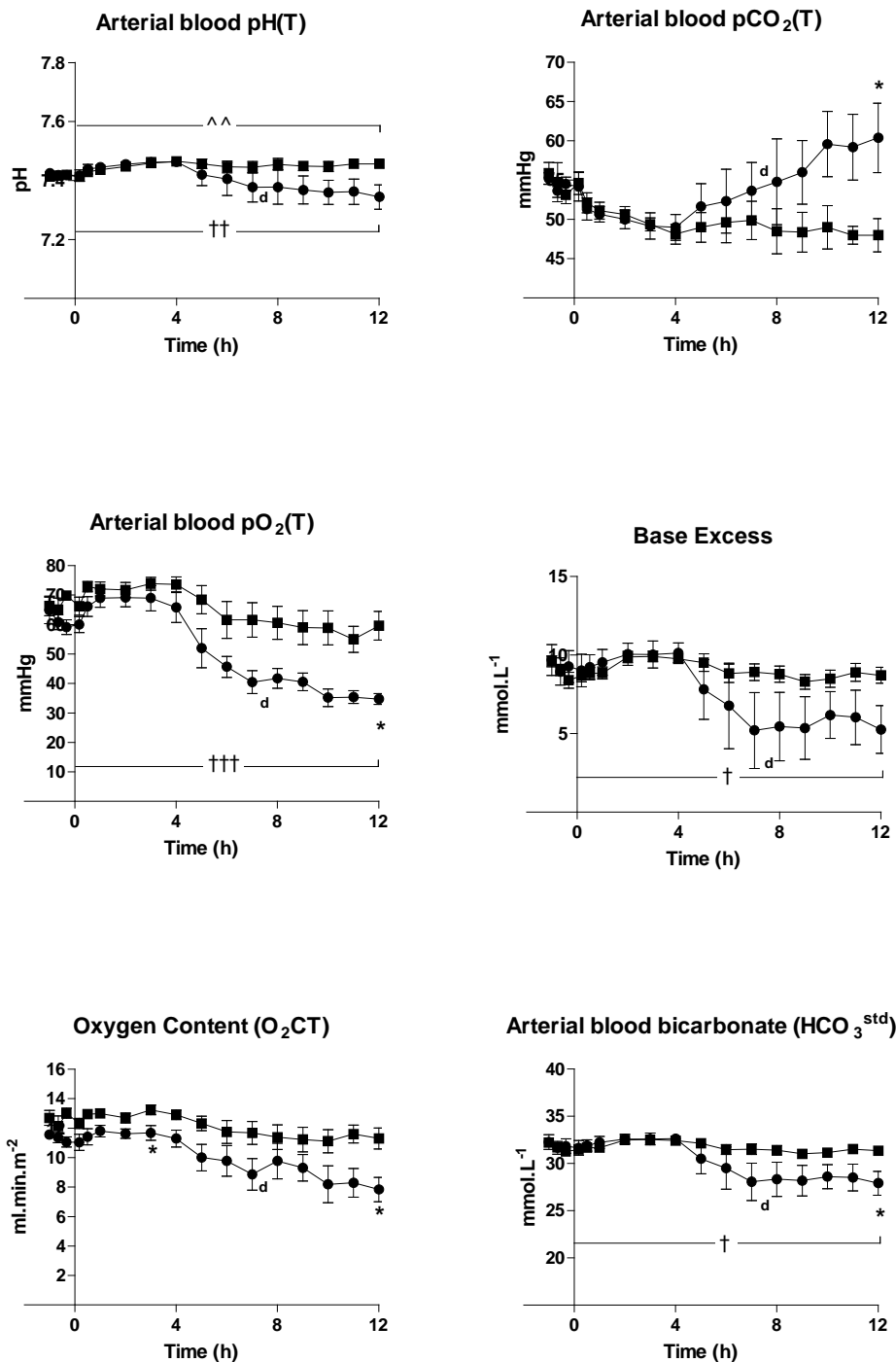


Figure 1- Arterial blood physiology. ● = HD Controls (n=6) and ■ = NAC Treated (n=8) 'd' represents a death in the HD control group. * $p < 0.05$ shows difference between HD control and NAC treated groups. ††† $p < 0.001$, †† $p < 0.01$ or † $p < 0.05$ indicate the statistical significance of changes from baseline levels in the HD control group at 12 h. ^ $p < 0.01$ indicates the statistical significance of changes from baseline levels in the NAC treated group at 12 h.

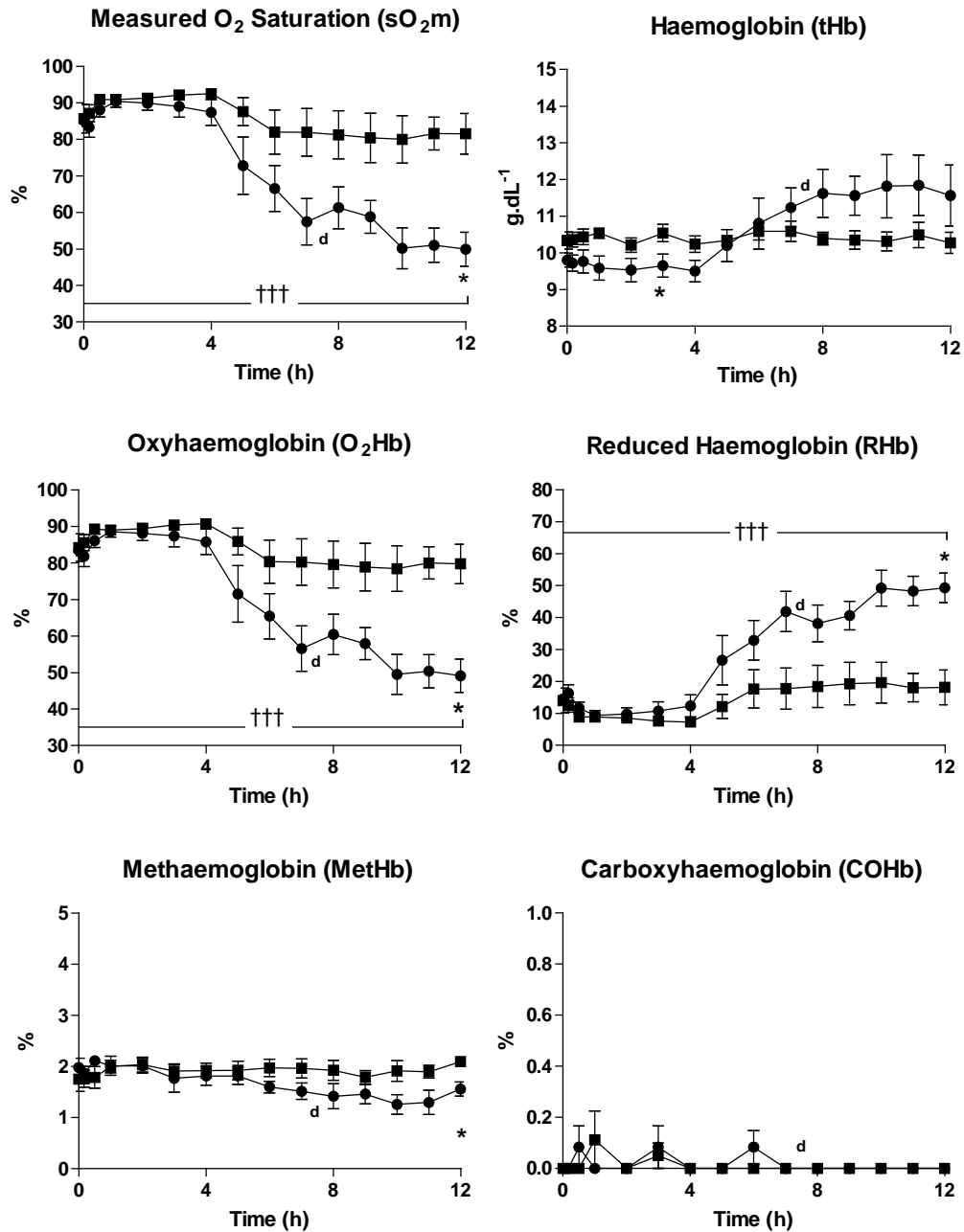


Figure 2 - Arterial blood co-oximetry. ● = HD Controls (n=6) and ■ = NAC Treated (n=8) 'd' represents a death in the HD control group. *p<0.05 shows difference between HD control and NAC treated groups. †††p<0.001 indicates the statistical significance of changes in HD control group at 12 h from baseline levels.

Arterial blood glucose concentration significantly increased over the course of the study in the HD control animals when compared to baseline values (Figure 3; †p<0.05). At 12 h the glucose concentration was significantly increased in the HD controls compared to the NAC treated animals (*p<0.05). In NAC treated animals there was no increase in glucose concentration with levels remaining normal.

Lactate concentration significantly increased over the course of the study in the HD control animals when compared to baseline values (Figure 3; † $p < 0.05$), but there was no significant difference seen between experimental groups.

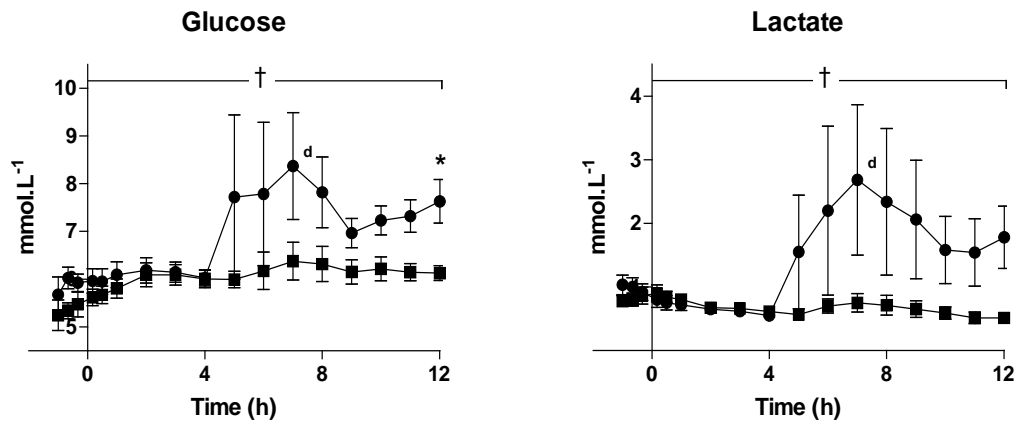


Figure 3- Arterial blood glucose and lactate concentrations. ● = HD Controls (n=6) and ■ = NAC Treated (n=8) 'd' represents a death in the HD control group. * $p < 0.05$ shows difference between HD control and NAC treated groups. † $p < 0.05$ indicates the statistical significance of changes from baseline levels in the HD control group at 12 h.

3.3.3 Cardiovascular parameters

At 12 h heart rate was significantly elevated in the HD controls compared to baseline levels (Figure 4; † $p < 0.05$). At 9 and 12 h heart rate was significantly higher in the HD control animals than in the NAC treated group (** $p < 0.01$ and * $p < 0.05$ respectively). NAC treated animals maintained a normal heart rate throughout the study. There were no other statistically significant differences between the groups in any other cardiovascular parameters.

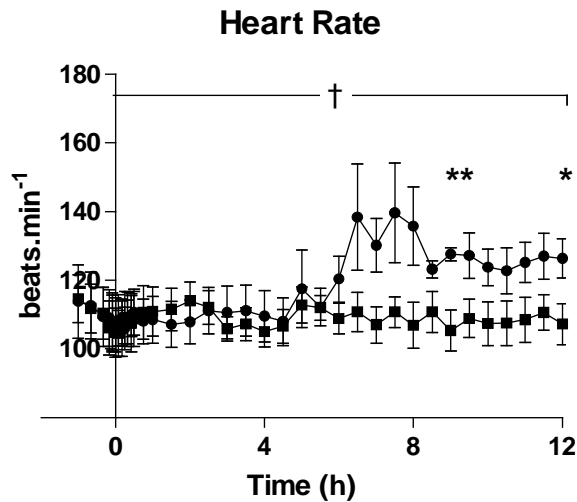


Figure 4 - Changes in heart rate. ● = HD Controls (n=6) and ■ = NAC Treated (n=8) 'd' represents a death in the HD control group. **p<0.01 and * p<0.05 show differences between HD control and NAC treated groups. †p<0.05 indicates the statistical significance of changes from baseline levels in the HD control group at 12 h.

3.3.4 Respiratory parameters

There were no significant differences between the HD control and NAC treated groups for any of the parameters investigated e.g. minute and tidal volume, lung resistance and compliance.

3.4 Calculated parameters

Shunt fraction was significantly increased at 12 h post exposure in the HD control group when compared to baseline levels (†††p<0.001) and when compared to NAC treated animals at the same time point (*p<0.05). The NAC treated group maintained shunt fraction at near normal baseline levels.

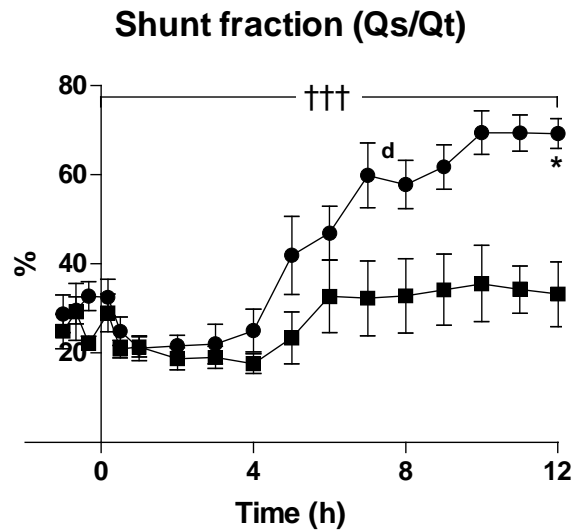


Figure 5- Changes in Shunt fraction: ● = HD Controls (n=6) and ■ = NAC Treated (n=8) 'd' represents a death in the HD control group. * p<0.05 shows difference between HD control and NAC treated groups. †††p<0.001 indicates the statistical significance of changes in HD control group at 12 h from baseline levels.

There was no significant change in EVLW over time in either group over the 12 h study period (Figure 6).

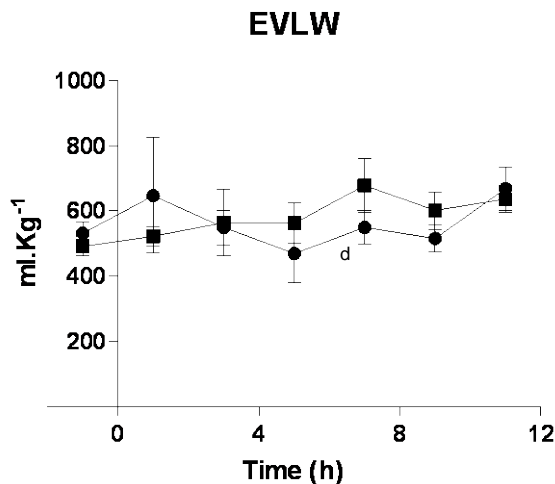


Figure 6 – Changes in extravascular lung water content. ● = HD Controls (n=6) and ■ = NAC Treated (n=8) 'd' represents a death in the HD control group.

3.5 Haematology

There were statistically significant increases in the number of red blood cells, reflected by the increased haemoglobin concentration and haematocrit in the HD control group when compared to baseline values at 12 h (††p<0.01 – Figure 7). When compared to the NAC treated group at 9 h these parameters were also

significantly different (* $p < 0.05$ for all parameters). NAC prevented the increase in red cell count, haematocrit and haemoglobin concentration maintaining near to historical air control values over the 12 h study [13]. No other changes in haematology parameters were noted.

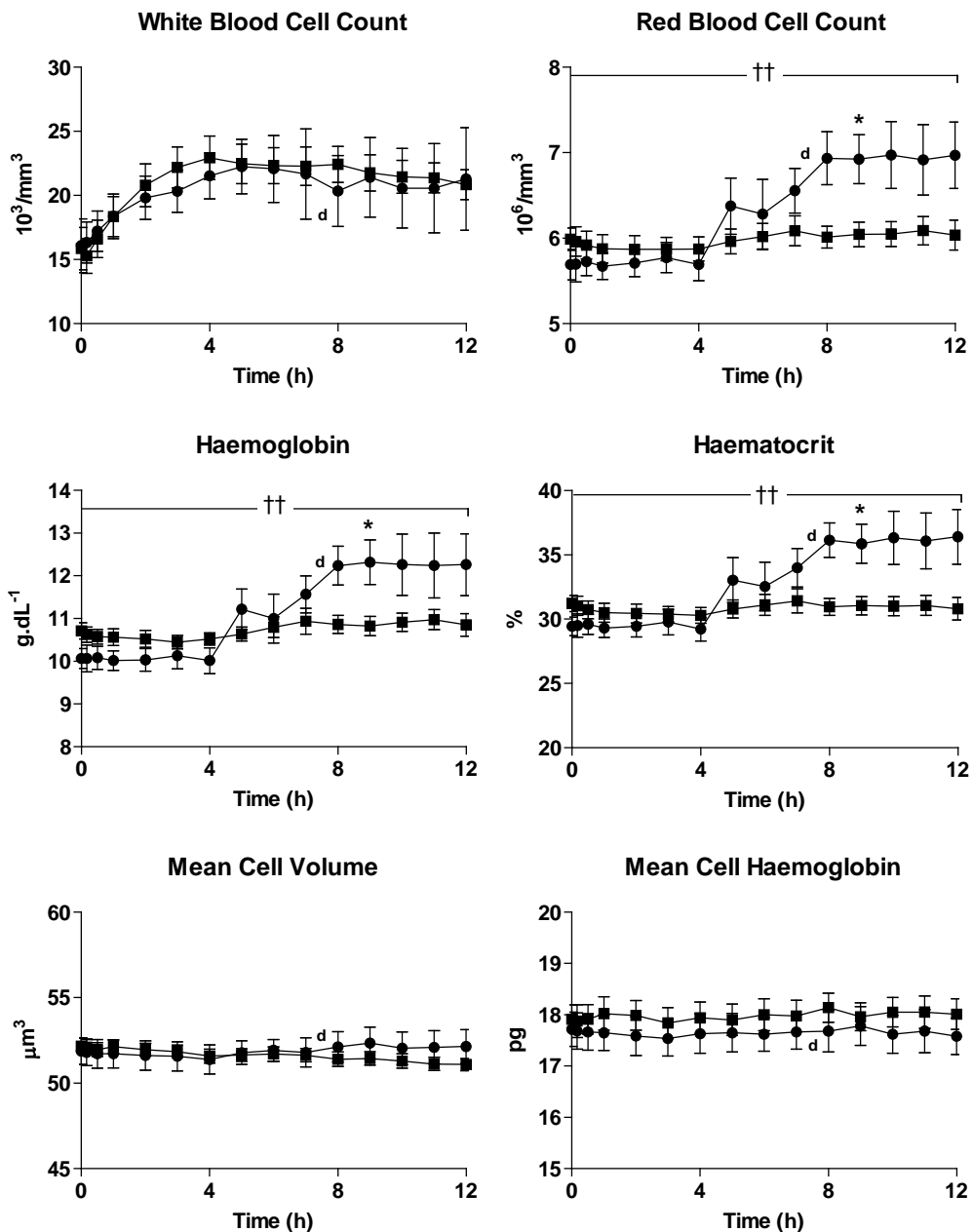


Figure 7 - Arterial blood haematology. ● = HD Controls (n=6) and ■ = NAC Treated (n=8) 'd' represents a death in the HD control group. * $p < 0.05$ shows difference between HD control and NAC treated groups. †† $p < 0.01$ indicates the statistical significance of changes in HD control group at 12 h from baseline levels.

3.6 Lavage Fluid Analysis

Differential WBC count analysis of terminal BAL fluid showed an increase in the number of neutrophils recruited to the lungs of HD control animals (Figure 8) compared to historical air controls (data not shown). NAC treated animals had a

significantly smaller percentage of neutrophils and concomitant higher percentage of lymphocytes in the lung compared to HD controls (*p<0.05).

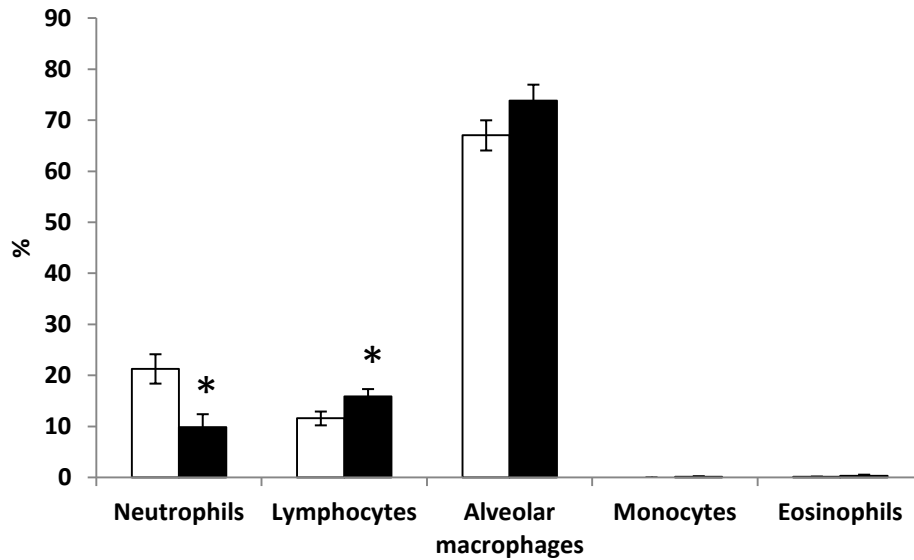


Figure 8 - Terminal BAL fluid differential WBC count: □= HD controls (n=5; no BAL from 1 animal) and ■ = NAC Treated (n=8). *p<0.05 shows differences between HD control and NAC treated groups.

The amount of protein in the terminal BAL fluid was increased in HD control animals compared to historical air controls (data not shown). NAC treated animals had a significantly lower concentration of protein compared to HD control animals (Figure 9; *p<0.05).

BAL fluid protein concentration

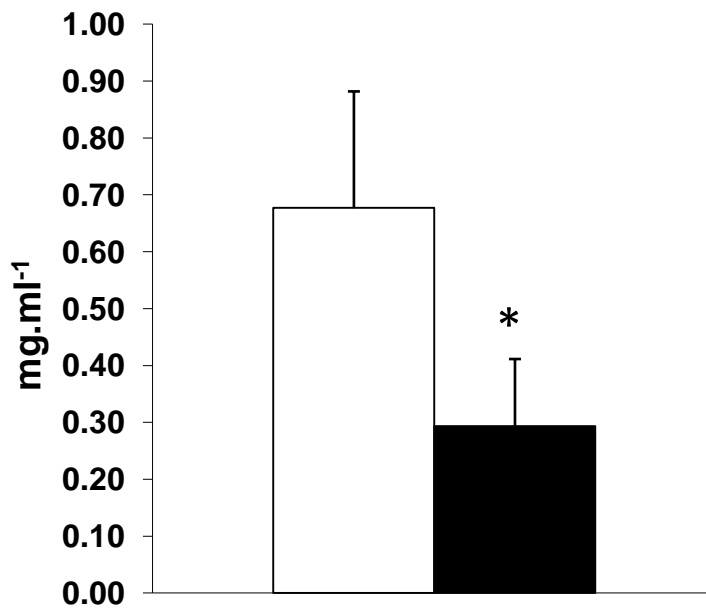


Figure 9 - Terminal BAL fluid protein concentration: □ = HD Controls (n=5; no BAL from 1 animal) and ■ = NAC Treated (n=8). *p<0.05 shows difference between HD control and NAC treated groups.

3.7 Measures of Lung Oedema and Inflammation

There was no significant difference in LWW:BW ratio between groups (Table 3).
There was no significant difference in the LWW:DW ratio between groups (Annex E).

Treatment	BW (Kg)	LWW (g) (- lavage)	LWW:BW ratio	Mean
HD Control	50.4	308.0	6.11	9.68 ± 1.06
HD Control	52.6	561.5	10.67	
HD Control	51.0	513.6	10.07	
HD Control	55.0	496.4	9.03	
HD Control	49.3	618.2	12.54	
HD + NAC	55.2	501.3	9.08	9.28 ± 0.39
HD + NAC	54.6	468.0	8.57	
HD + NAC	51.2	419.7	8.20	
HD + NAC	50.0	493.7	9.87	
HD + NAC	50.2	484.2	9.65	
HD + NAC	55.8	455.9	8.17	
HD + NAC	54.2	623.3	11.50	
HD + NAC	52.4	482.7	9.21	

Table 3: LWW:BW ratios. Note n=5 for HD control group as no BAL fluid could be obtained from one animal.

3.7.1 Inflammatory mediators

ELISA analysis revealed an inconsistent response to the presence of the inflammatory mediators, IL-1 β , IL-6, IL-8 and TNF α across all animals in BAL fluid

(Annex E). Annex E shows the comparison of cytokine production for the HD controls and NAC treated groups. Whilst it appears that the NAC may have a dampening effect on cytokine production (IL-6 and IL-8), the lack of reproducibility across animals in the HD control group means that no conclusion can be supported.

3.7.2 Markers of neutrophil activation

A significant increase in urinary desmosine was noted at 4 hours post exposure in HD control animals (Annex D). NAC treated animals prevented the early increase in urinary desmosine, however, there was no difference between groups over the course of the study. No significant differences were detected in BAL fluid desmosine levels between groups. BAL fluid MPO, NE and MMP activity were also not significantly different between groups however there was a downward trend for all markers in the NAC treated animals.

3.8 Urine Adducts

The HD metabolite MSMTESE could be detected from 4 h in the urine of both experimental groups before increasing rapidly from 6 h (Figure 10). There was no difference in the amount of MSMTESE present in the urine over time between the HD control and NAC treated groups.

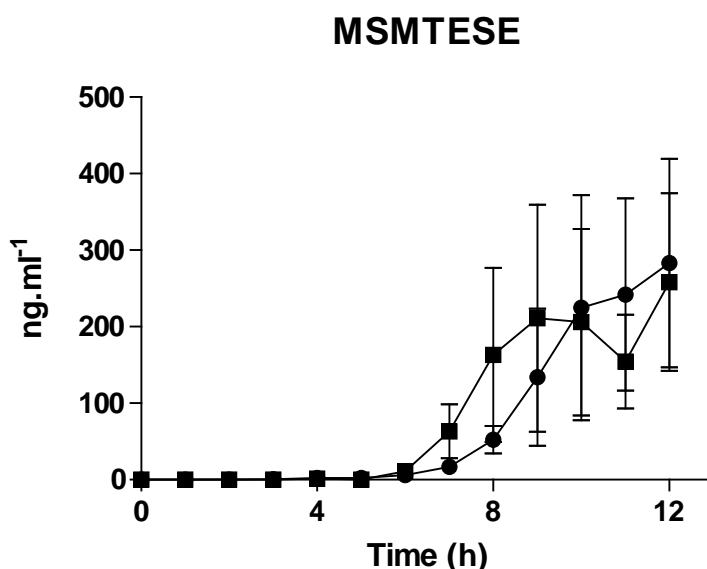


Figure 10 - Concentrations of MSMTESE in the urine following exposure. ● HD controls (n=6) ■ NAC Treated (n=8).

3.8.1 Lung tissue thiols

Total lung tissue thiol concentrations were significantly increased in the NAC treated animals when compared to HD controls (Figure 11; *p<0.003) independent of lobe effect or section effect.

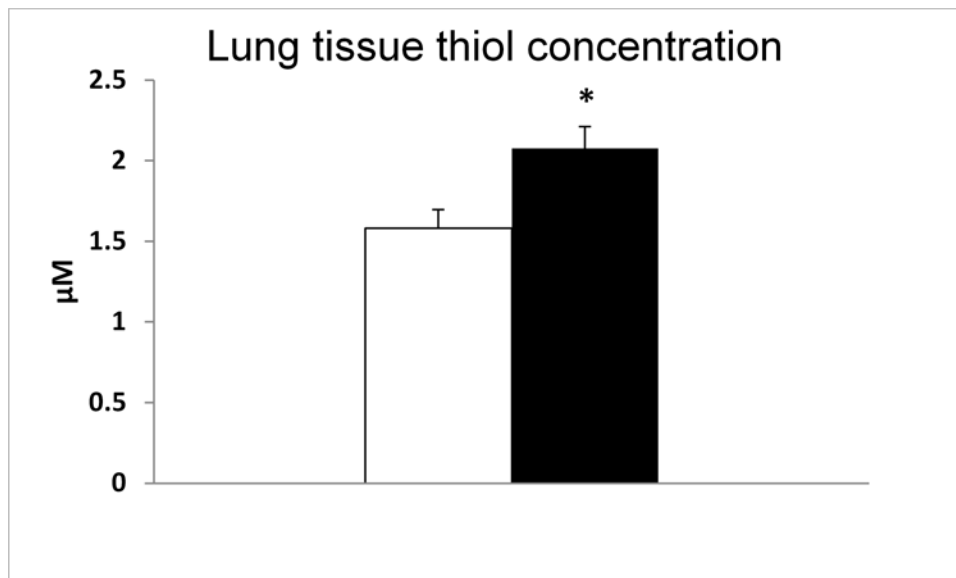


Figure 11 – Total tissue thiol concentration in the lung, pooled across all lobes (left and right apical, medial and caudal) for □ = HD Controls (n=6) and ■ = NAC Treated (n=8). * $p < 0.003$ indicates significant increase in thiol concentration in the NAC treated animals.

3.9 Gross Pathology and Histopathology

3.9.1 Gross Pathology

At post mortem there was no discernible difference in the position of the ET tube whether placed via the mouth or tracheostomy.

Tracheal rings taken from above and below the tip of the ET tube were compared visually for both HD control and NAC treated animals. Tracheal rings taken from above the tip of the ET tube in both groups (therefore protected from the HD injury) were indistinguishable from historical air exposed animals' samples taken from both above and below the ET tube [13]. Damage was therefore confined to the areas below the ET tube in both groups, with evidence of inflammation (Figure 12). These effects were visually less pronounced in the NAC treated animals.



HD Control



NAC Treated

Figure 12 - Tracheal rings taken from below the end of the ET tube comparing HD control with NAC treated animal.

In HD control animals there appeared to be extensive HD-induced damage to the bronchi when compared to the NAC treated animals. The presence of excessive mucus and epithelial sloughing were also more apparent. Figure 13 shows the gross pathology of the main bronchi from an animal in each of the experimental groups.

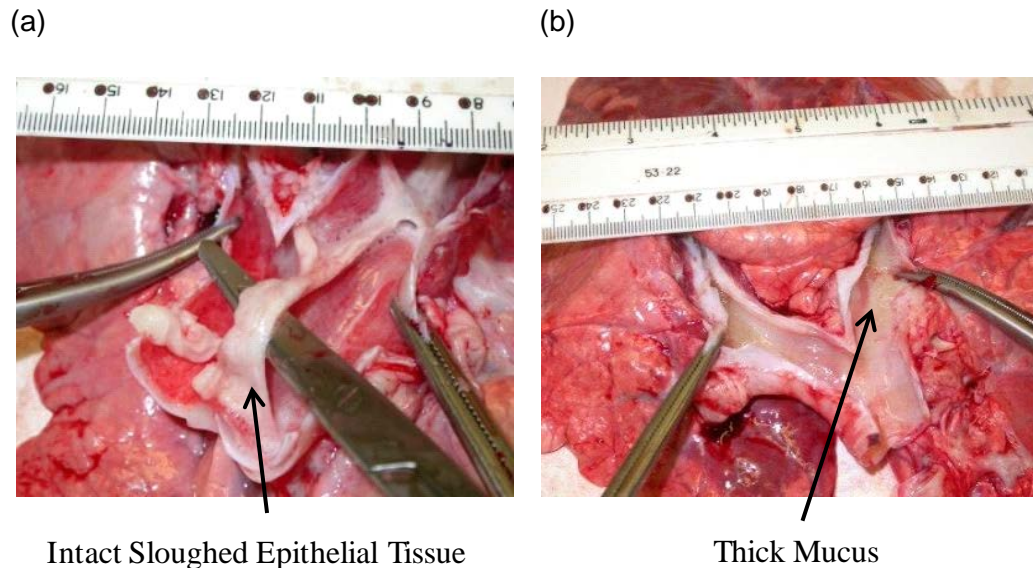


Figure 13 - Gross pathology of main bronchi in (a) HD control and (b) NAC treated animals.

The estimation of the extent of the damage to the lungs as a whole, judged by gross pathology, was highly subjective but the NAC treated group appeared to have less damage than the HD control group (Figure 14).

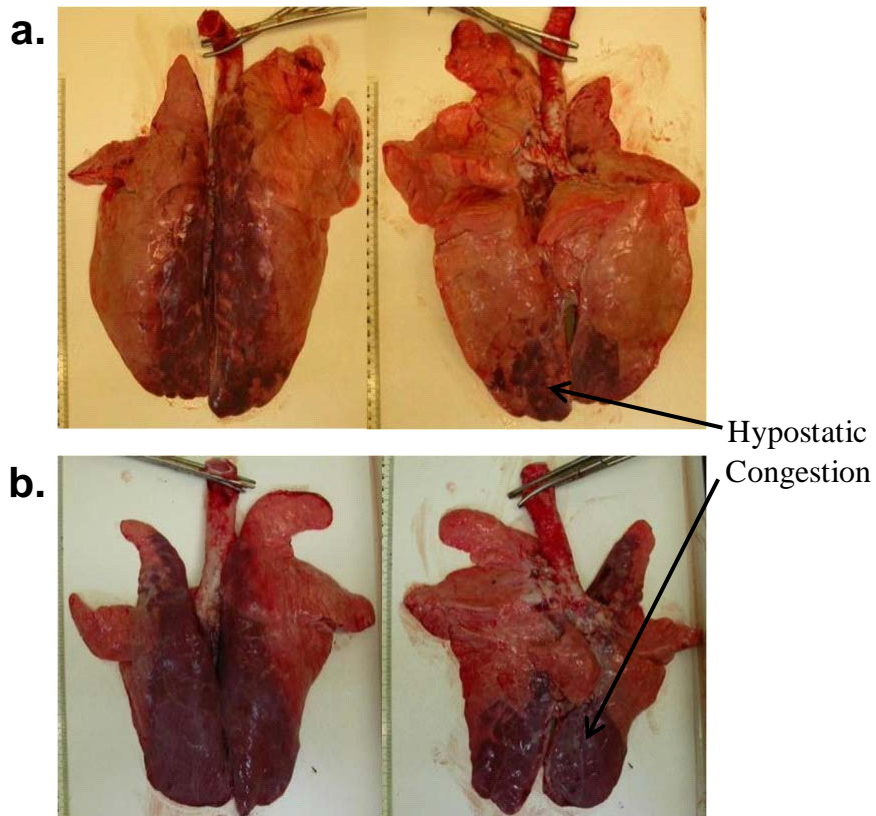


Figure 14 - Gross pathology of lungs in (a) HD control and (b) NAC treated animals. Each picture shows lungs from posterior (L) and anterior (R) views.

3.9.2 Histopathology

The average histopathology scores for the lungs were not significantly different between groups (Table 4).

Group	No. of Animals	Average Score \pm SEM
HD Controls	6	34.50 \pm 1.77
NAC Treated	8	27.63 \pm 1.57

Table 4. Comparison of average lung scores for HD control and NAC treated groups.

Figure 15 shows the tracheal epithelium for a HD Control (a) and NAC treated animal (b). Damage to the epithelium is apparent in both groups, although less severe in the NAC treated group.

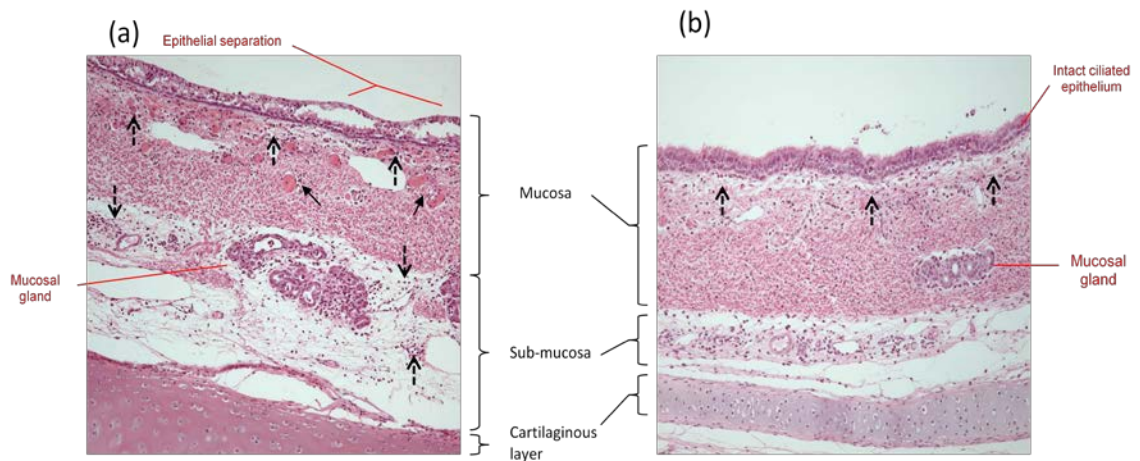


Figure 15: Tracheal epithelium for HD Control (a) showing intra-epithelial separation, blood vessel congestion (↑), tissue oedema and inflammatory cell infiltration (↑). NAC treated (b) animal displaying region of intact ciliated epithelium, sub-epithelial oedema and inflammatory cell infiltration. Haematoxylin & eosin stained sections (original magnification 100×).

All additional data not reported in the main text will be incorporated in Annex E.

4 Discussion

We previously reported the results of the 12 h toxicology study [13] in which a reproducible model of exposure to inhaled HD was described. The animal model data are consistent with clinical descriptions reported following human exposure to HD [10,19,20]. This model has now been used to assess treatment efficacy, the results of which are reported here.

This study has shown that, at 12 h post exposure, the HD control animals had developed an ALI which manifest as a decreased arterial blood oxygenation (PaO_2 , arterial blood saturation), bicarbonate concentration and base excess, with increased arterial blood pCO_2 when compared to baseline values. This confirms the presence of a respiratory acidosis in these animals. The shunt fraction was significantly increased at 12 h compared to baseline values and the heart may be compensating for this as indicated by the significantly increased heart rate at the same time point. There were no changes in any of the respiratory parameters measured following HD exposure.

Compared with historical air control animals (data not shown) markers of lung oedema (EVLW, LWW:BW and LWW:DW ratios) remained unchanged at 12 h however there was an increase in protein concentration and number of neutrophils in terminal BAL fluid. An accumulation of neutrophils in air spaces is consistently seen in ALI in both humans and in animal models [21] and it is postulated that they contribute either directly or indirectly to lung injury [22].

Post exposure treatment with multiple inhaled doses of nebulised NAC was effectively able to reverse or reduce a number of the physiological effects of HD exposure. These include significantly improved arterial blood oxygenation, reduced heart rate and shunt fraction, as well as other markers of lung injury (significantly reduced neutrophil numbers and protein concentration in terminal BAL fluid).

There were no changes in any of the respiratory parameters, or markers of lung oedema, measured in the treatment group.

Pathological examination revealed HD-induced damage confined to the areas below the ET tube in both experimental groups, with areas of inflammation, epithelial sloughing and mucus production. Early local effects on epithelial tissues occur due to sulphur mustard's ability to penetrate cell membranes rapidly [23]. However, these effects were visually less pronounced in the NAC treated animals, where there appeared to be a decrease in the subjective severity score of the HD injury observed at *post mortem*. The average lung scores were lower for NAC treated animals than for HD controls which may suggest a further beneficial role for NAC in reducing HD-induced lung pathology. We speculate that a combination of inflammatory changes to the trachea and epithelial sloughing of the major bronchi, result in debris within the airways. This has the potential to move and block smaller airways. Reduced ability to clear debris and increased build up of mucus may result from loss of the mucociliary escalator or its function. Potentially this combination of events may be responsible for reduced gas exchange resulting in decreased arterial blood oxygenation and increased shunt fraction through ventilation/perfusion mismatch.

NAC is known to have mucolytic and anti-oxidant properties and, as a synthetic derivative of cysteine, has a role as a glutathione donor. HD exposure is associated with inflammatory and oxidative injury causing damage in both the upper and lower

respiratory tract. It has been postulated to deplete reduced GSH levels [24]. Glutathione depletion and oxidative stress are key events contributing to cell death. Decreased cell matrix adhesion, increased mucus production and loss of ciliary function have all been reported following HD exposure *in vitro* [25]. It has not been possible to fully elucidate the beneficial mechanism of action of NAC in this study; however, our results show a significant reduction in neutrophil infiltration into the lungs, indicating an anti-inflammatory effect of NAC. NAC has been reported to down regulate the activity of the AP-1 transcription factor, contributing to reduced infiltration of inflammatory cells into alveolar spaces, although this study used the half mustard analogue CEES, rather than HD [26]. The ability of NAC to protect against HD-induced lung injury could also be explained by both its ability to raise intracellular concentrations of cysteine (as shown in our study), and hence of GSH, and/or by scavenging oxidant species [15]. NAC is widely used as a mucolytic drug, reducing viscosity and elasticity of mucus, due to its ability to reduce disulphide bonds [16]. NAC has also been shown to ameliorate the symptoms of chronic lung injury, including cough and dyspnoea, in human survivors many years after HD exposure, an effect likely related to its combined anti-inflammatory and anti-oxidant activities [27].

This study has demonstrated the beneficial effects of multiple inhaled doses of NAC on a number of clinically relevant physiological parameters for up to 12 hours following inhaled HD exposure. We have shown a significantly reduced number of neutrophils in the terminal BAL fluid of NAC treated animals, as well as a significantly increased lung tissue thiol concentration compared with HD controls. However, we are unable to state whether inhaled HD reduced tissue thiol concentrations compared with air exposed control animals. Irrespective of mechanism, NAC improved a number of key clinically relevant physiological indices affected by exposure to HD and should be further investigated as a potential commercial off the shelf (COTS) treatment for HD-induced ALI. The NAC dosing protocol for this study was taken from human dosing protocols in consultation with medical colleagues. It is not possible to state whether optimization of this protocol may provide a more effective treatment strategy for this injury; however, further studies should investigate the potential effectiveness of a multi pharmacologic approach, targeting a number of cellular responses.

5 Conclusions and Recommendations

Exposure to inhaled HD in terminally anaesthetised spontaneously breathing large white pigs resulted in an ALI, with physiological and pathological changes consistent with those described in human poisoning [20,23].

Post-exposure therapy with multiple inhaled dose of NAC reduced key clinically relevant physiological parameters of inhaled HD-induced lung injury for up to 12 hours after exposure. This provides greater confidence to the findings of Anderson [3] and better extrapolation to man.

It is recommended that further studies are conducted in this model to investigate the efficacy of multiple inhaled therapies (incorporating NAC) to further restore the physiological indices in HD-induced ALI.

6 Acknowledgements

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C. Masey for surgical preparation of the subjects; Animal Services Staff for animal husbandry; C. Taylor for histopathological analyses; R. Read for urinary analysis of metabolites; G. Allan and J. McCarthy for tissue thiol analysis and R. Mansson and R. Gwyther for statistical analysis; Dr J. Shute, University of Portsmouth, for the analysis of markers of neutrophil activation.

7

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ANNEX A Characterisation of the exposure system using the Gasmet™

The aim of the characterisation was to determine whether a real time method for measuring HD vapour concentration could be incorporated into the existing HD exposure system. It had been purported that a Gasmet™ Fourier Transform Infra-red (FTIR) Spectrometer would be a suitable and improved method for HD vapour concentration determination compared to the off-line Porapak analysis used previously.

A system was devised which incorporated the Gasmet™ into the existing exposure system (Fairhall *et al*; 2008) such that HD vapour samples could be analysed in real time via the sampling port upstream of the exposure port. This involved redesign of the nitrogen carrier gas system to support the Gasmet™, setting up chemical warfare agent reference libraries and associated interferences. Advice was also sought on the interpretation of sample spectra for HD vapour by the manufacturer of the Gasmet™ to ensure accurate measurement.

A series of experiments were carried out. These sought to address whether the Gasmet™ was suitable for determining real time HD vapour concentration; if so, that it was accurate and reproducible across the range of concentrations likely to be encountered during the treatment study.

- Measuring HD vapour output

The water bath temperature was set to 38.2 °C and the Porton vapour generators (PVGs) allowed to equilibrate for 30 min. The temperature chosen was based on historical data from the previous HD dose ranging study. The Gasmet™ was incorporated into the exposure system to sample HD vapour from the exposure line, upstream of the exposure port (from where the animal is exposed). Porapak tubes were positioned upstream and downstream of the exposure port for off-line analysis and comparison. From historical data, at this temperature, an average target concentration of 70 µg.l⁻¹ over a 10 min exposure period was expected. Three ten min exposures were carried out with time allowed for complete evacuation of HD vapour from the system between exposures.

- Assessing range of system output

In addition to measuring the output at 38.2 °C, a further two temperatures (30 °C and 42 °C) were assessed to investigate the range of concentrations that could be achieved by the exposure system. The Gasmet™ was used to sample upstream; Porapak tubes were used to sample both upstream and downstream of the exposure port. Two runs at each temperature were carried out.

- Comparison of Gasmet™ derived concentration with Porapak derived concentration.

The HD vapour concentration obtained from the Gasmet™ was compared to the concentration obtained by Porapak by sampling both upstream and downstream of the exposure port in two separate ten min exposures. The water bath temperature was set at 38.2 °C.

A-1 Results of Characterization using Gasmet™

- Measuring HD vapour output

Following the three ten min exposures, the HD vapour concentration generated by the exposure system as determined by Gasmet™ from the upstream sampling site was 70.07 ± 0.28 µg.l⁻¹. This concentration was comparable to outputs generated

during the previous 12 h toxicology study at this temperature. The concentration obtained by Porapak from the same site was $67.94 \pm 13.4 \mu\text{g.l}^{-1}$.

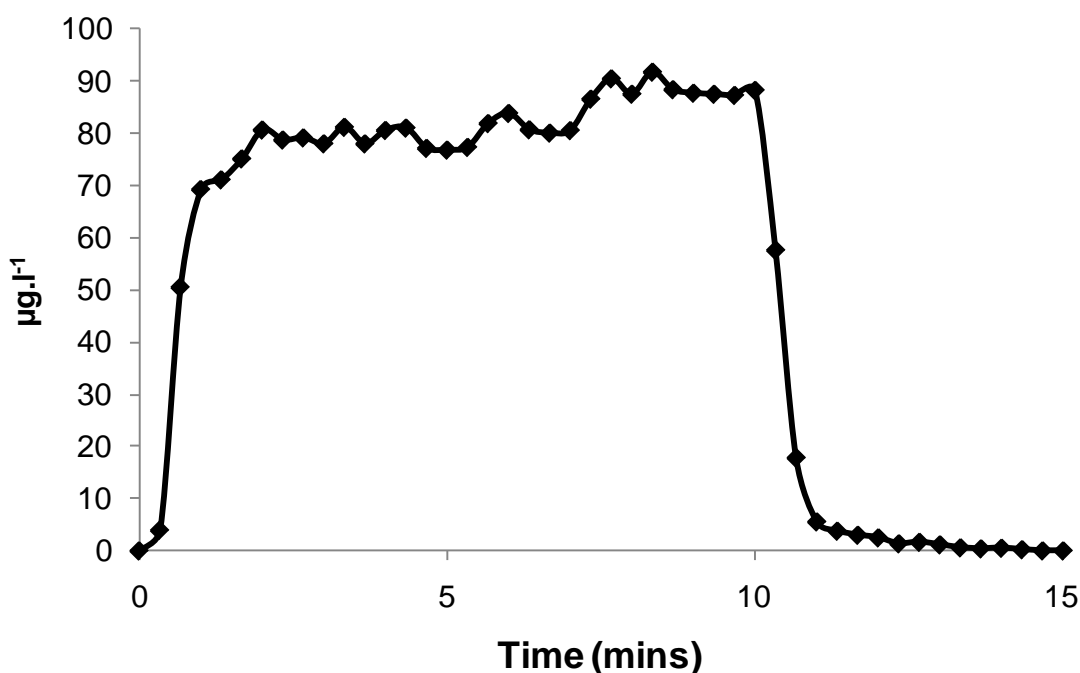


Figure 1: Graph showing typical HD vapour concentration ($\mu\text{g.l}^{-1}$) over time (mins) obtained by the Gasmeter™ during animal exposure. The profile clearly demonstrates that the target HD concentration was rapidly achieved upon turning the HD generators on, was maintained with an acceptable level of stability, and rapidly decayed to zero when the generators were switched off.

- Assessing range of HD vapour output

The minimum concentration obtained from the upstream sampling site by Gasmeter™ was $37.95 \mu\text{g.l}^{-1}$ following a ten min exposure at a temperature of 30.0°C when the PVGs were set at a flow rate of 2 l.min^{-1} (Table 1). The maximum concentration obtained at 42°C was $83.85 \mu\text{g.l}^{-1}$ following a ten min exposure when the PVGs were set at a flow rate of 2 l.min^{-1} . Increasing the flow rate through the PVGs to 4 l.min^{-1} achieved a peak concentration in excess of $100 \mu\text{g.l}^{-1}$. This is outside of the calibrated range of the Gasmeter™ for HD vapour and it is not anticipated that we would need to reach this concentration during the treatment study.

Temperature ($^\circ\text{C}$)	Concentration by Gasmeter™ ($\mu\text{g.l}^{-1}$)	Concentration by Porapak upstream ($\mu\text{g.l}^{-1}$)	Concentration by Porapak downstream ($\mu\text{g.l}^{-1}$)
30.0	37.95 ± 0.92	37.99 ± 1.04	41.52 ± 1.82
38.2	70.07 ± 2.83	67.94 ± 13.33	91.87 ± 3.17
42.0	83.85 ± 6.72	79.80 ± 20.34	108.33 ± 2.97

Table 1: Comparison of Gasmeter™ derived HD vapour concentration with Porapak derived concentration.

There was good agreement in the concentrations measured by the Gasmeter™ with the Porapak analysis at the upstream sampling site. The consistency of the HD vapour produced by the exposure system was also assessed by switching sampling of the Gasmeter™ from the upstream to the downstream sampling site during HD vapour generation. This found that there was no discernible difference between the two sampling sites.

ANNEX B

Histopathology

Your Ref: HD47, HD48, HD50, HD51,
HD52, HD53, HD54, HD55, HD56, HD57,
HD58, HD59, HD60, HD61, HD62
Our Ref: 11/42889-11/42904

Sender: Chris Taylor

Location: DSTL. Porton Down
Salisbury, Wiltshire SP4 0JQ

Date: 04.08.2011

Pathology Report

Gross Observations

- Pathology assessment of lung injury in an anaesthetized porcine model of sulphur mustard (HD) inhalation. H&E stained spleen, liver, heart, kidney, lymph node, thymus, trachea and lung tissue sections from right and left apical, medial and caudal lobes.

A scoring system has been developed to assess the lung pathology. The parameters that have been measured are the following:

- Bronchiolar epithelial degeneration and necrosis
- Hypertrophy/Hyperplasia of bronchiolar epithelium
- Cell debris in the bronchiolar lumen
- Perivascular (and peribronchiolar) oedema, fibrin and cell infiltrates
- Alveolar oedema and fibrin
- Alveolar cell infiltrates
- Interstitial (septal) oedema
- Haemorrhages

Parameters were evaluated as follows:

0: Not observed

1: Mild

2: Moderate

3: Severe

WNL: *Within Normal Limits*

Microscopic Observations

Results for lung pathology are summarised in the attached Table (.xls file)

Other findings:

HD47 11/42893

Spleen: WNL

Liver: WNL

Heart: WNL

Kidney: Mild infiltration of mononuclear cells

Lymph node: WNL

Thymus: WNL

Trachea: Hyperaemia. Loss of epithelium

HD48 11/42892

Spleen: WNL
Liver: Minimal hepatitis
Heart: WNL
Kidney: Mild infiltration of mononuclear cells
Lymph node: WNL
Trachea: Hyperaemia. Subepithelial oedema

HD50 11/42891

Spleen: WNL
Liver: Minimal hepatitis
Heart: WNL
Kidney: Mild infiltration of mononuclear cells
Lymph node: WNL
Thymus: WNL
Trachea: Hyperaemia. Loss of epithelium

HD51 11/42890

Spleen: Mild lymphoid depletion
Liver: Severe centrilobular necrosis
Heart: Scattered necrotic cardiomyocytes
Kidney: WNL
Lymph node: WNL
Trachea: Very severe hyperaemia. Submucosal oedema and loss of epithelium

HD52 11/42889

Spleen: Mild lymphoid depletion
Liver: Mild periportal hepatitis
Heart: WNL
Kidney: Mild infiltration of mononuclear cells
Lymph node: WNL
Upper trachea: Hyperaemia
Lower trachea: Hyperaemia

HD53 11/42904

Spleen: WNL
Liver: WNL
Heart: WNL
Kidney: WNL
Lymph node: WNL
Upper trachea: Hyperaemia. Sloughed epithelial cells and mucous plug
Lower trachea: Hyperaemia. Sloughed epithelial cells and mucous plug

HD54 11/42903

Spleen: WNL
Liver: WNL
Heart: WNL
Kidney: WNL
Lymph node: WNL
Upper trachea: Hyperaemia. Sloughed epithelial cells and mucous plug
Lower trachea: Hyperaemia. Sloughed epithelial cells and mucous plug. Artefactual loss of epithelium

HD55 11/42902

Spleen: WNL
Liver: Periportal infiltrates
Heart: WNL
Kidney: WNL
Lymph node: WNL
Upper trachea: Hyperaemia
Lower trachea: Hyperaemia. Sloughed epithelial cells

HD56 11/42901

Spleen: WNL
Liver: Periportal infiltrates
Heart: WNL
Kidney: WNL
Lymph node: WNL
Upper trachea: Hyperaemia. Sloughed epithelial cells and mucous plug
Lower trachea: Hyperaemia.

HD57 11/42900

Spleen: Mild lymphoid depletion
Liver: WNL
Heart: WNL
Kidney: WNL
Lymph node: WNL
Upper trachea: Hyperaemia. Loss of epithelium
Lower trachea: Hyperaemia. Loss of epithelium

HD58 11/42899

Spleen: WNL
Liver: WNL
Heart: WNL
Kidney: WNL
Lymph node: WNL
Upper trachea: WNL
Lower trachea: WNL

HD59 11/42898

Spleen: Mild lymphoid depletion
Liver: Mild hepatitis and centrilobular necrosis
Heart: WNL
Kidney: WNL
Lymph node: WNL
Upper trachea: Loss of epithelium
Lower trachea: Hyperaemia. Loss of epithelium

HD60 11/42897

Spleen: Moderate lymphoid depletion
Liver: Mild hepatitis (periportal infiltrates)
Heart: WNL
Kidney: WNL
Lymph node: WNL
Upper trachea: Hyperaemia. Sloughed epithelial cells and mucous plug
Lower trachea: Hyperaemia. Submucosal oedema. Sloughed epithelial cells and mucous plug

HD61 11/42896

Spleen: WNL
Liver: Mild hepatitis (periportal infiltrates)
Heart: WNL
Kidney: WNL
Lymph node: WNL
Upper trachea: Hyperaemia. Sloughed epithelial cells and mucous plug
Lower trachea: Hyperaemia. Sloughed epithelial cells and mucous plug

HD62 11/42894

Spleen: WNL
Liver: Mild hepatitis (periportal infiltrates)
Heart: WNL
Kidney: WNL
Lymph node: WNL
Upper trachea: Hyperaemia. Sloughed epithelial cells and mucous plug
Lower trachea: Hyperaemia. Sloughed epithelial cells and mucous plug

Diagnosis

Not applicable

Remarks

There are some lung lobes with 2 tissue samples. The most affected one is used for the score.

There is no LA sample from animal HD50

Pathologist Signature:
MRCVS

Name: F. Javier Salguero, DVM, PhD,

Date: 04.08.2011

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Animal	HD 47						HD 48						HD 50						HD 51						HD 52						HD 53						HD 54																																			
Group																																																																								
Lung lobe	LA	RA	LM	RM	LC	RC	LA	RA	LM	RM	LC	RC	LA	RA	LM	RM	LC	RC	LA	RA	LM	RM	LC	RC	LA	RA	LM	RM	LC	RC	LA	RA	LM	RM	LC	RC	LA	RA	LM	RM	LC	RC																														
Brochiolar epithelial degeneration and necrosis	1	0	0	1	0	0	0	0	1	1	0	0		0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	0	1	1	1	1	1																														
Hypertrophy/Hyperplasia of bronchiolar epithelium	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0																														
Cell debris in bronchiolar lumen	0	1	0	0	1	0	0	0	0	0	1	1		1	0	0	1	1	1	1	1	1	1	1	2	1	2	1	3	1	2	1	1	1	1	1	1	1	1	1	1	1	1																													
Perivascular oedema, fibrin and cell infiltrates	0	0	0	0	1	1	1	0	1	1	0	0		1	1	0	0	0	2	2	2	2	1	2	1	1	3	2	3	2	0	1	0	1	0	1	1	1	1	2	0	1																														
Alveolar oedema and fibrin	0	0	0	0	0	1	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0																														
Alveolar cell infiltrates	0	0	0	1	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	1	0	1	0	0	2	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0																														
Interstitial oedema	1	0	2	2	1	1	1	0	0	0	0	0		1	1	2	1	1	2	3	3	3	2	2	1	1	3	1	3	2	0	0	0	2	0	1	0	0	1	2	0	0																														
Haemorrhages	0	0	0	0	0	1	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0																													
Sum	2	1	2	4	3	4	2	0	2	2	1	1	0	3	3	2	3	3	6	7	7	8	6	9	4	5	11	10	9	9	4	3	2	6	2	4	2	4	4	7	3	3																														
	2.7						1.3						2.8						7.2						8.0						3.5						3.8																																			

ANNEX C

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Analytical Chemistry Report

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Customer:	S Rudall
Issue Date:	14 December 2011
Customer Reference	Thiol analysis of Pig Lung homogenates
Report. No.	1112844
Project Code:	701012_1000
Sample Description:	Pig lung homogenates HDP47 to HDP 62
Customer Sample Numbers:	HDP47 (LA;LM;LC;RA;RM;RC) to HDP 62 (LA;LM;LC;RA;RM;RC)
Customer Requirements:	Quantitation of thiols
Analytical Services Sample Numbers:	1112844-1112933
Principal Analyst:	J McCarthy and G Allan
Analytical Method(s) used: <i>(In-house Method Number)</i>	Commercial kit used: Molecular Probes, Thiol and Sulfide Quantitation Kit (T6060)
Date of Receipt of Sample(s):	01 June 2011
Date of Analysis:	01 December 2011

All the samples referred to in this report were analysed as received.
For further information or queries please contact the Chemical Analysis Co-ordinator on the above telephone number.

Report Authorised by	<u>J. Riches</u>	Position:	<u>Technical head</u>
Signature	<u>[Signature]</u>	Date:	<u>14/01/12</u>

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Analytical Details

The analysis was performed using the Molecular Probes' Thiol and Sulfide Quantitation Kit (T-6060). The procedure is attached at the end of this report (Annex A).

Initial analyses using neat samples suggested that dilution was necessary, therefore samples were re-run and diluted 20 × in Buffer A prior to analysis.

The samples were run in three batches: (1) HDP 47, 48, 50, 51, 52; (2) HDP 53-57; (3) HDP 58-62.

For each batch, a fresh L-cysteine working solution was prepared and calibrated using Ellman's reagent as per the instructions. These working solutions were used to generate standard curves against which the sample thiol concentrations were determined.

The addition of L-BAPNA was timed at 30 second intervals and the absorbance measurements were similarly timed.

Analytical Results

LSSN	Customer Ref	Dilution factor	Spectrometer reading	Corrected Abs	[THIOL] μM
1112844	HDP47 LA homogenate	20	0.4265	0.2322	1.5
1112845	HDP47 LM homogenate	20	0.4589	0.2646	1.7
1112846	HDP47 LC homogenate	20	0.4542	0.2599	1.6
1112847	HDP47 RA homogenate	20	0.4471	0.2528	1.6
1112848	HDP47 RM homogenate	20	0.437	0.2427	1.5
1112849	HDP47 RC homogenate	20	0.5443	0.3500	2.2
1112850	HDP48 LA homogenate	20	0.5318	0.3375	2.1
1112851	HDP48 LM homogenate	20	0.5259	0.3316	2.1
1112852	HDP48 LC homogenate	20	0.5701	0.3758	2.3
1112853	HDP48 RA homogenate	20	0.5847	0.3904	2.4
1112854	HDP48 RM homogenate	20	0.371	0.1767	1.1
1112855	HDP48 RC homogenate	20	0.436	0.2417	1.5
1112856	HDP50 LA homogenate	20	0.4849	0.2906	1.8
1112857	HDP50 LM homogenate	20	0.5174	0.3231	2.0
1112858	HDP50 LC homogenate	20	0.5261	0.3318	2.1
1112859	HDP50 RA homogenate	20	0.5371	0.3428	2.1
1112860	HDP50 RM homogenate	20	0.4061	0.2118	1.3
1112861	HDP50 RC homogenate	20	0.5941	0.3998	2.5

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1112862	HDP51 LA homogenate	20	0.4609	0.2666	1.7
1112863	HDP51 LM homogenate	20	0.5222	0.3279	2.0
1112864	HDP51 LC homogenate	20	0.4798	0.2855	1.8
1112865	HDP51 RA homogenate	20	0.5458	0.3515	2.2
1112866	HDP51 RM homogenate	20	0.3688	0.1745	1.1
1112867	HDP51 RC homogenate	20	0.548	0.3537	2.2
1112868	HDP52 LA homogenate	20	0.4824	0.2881	1.8
1112869	HDP52 LM homogenate	20	0.535	0.3407	2.1
1112870	HDP52 LC homogenate	20	0.364	0.1697	1.1
1112871	HDP52 RA homogenate	20	0.6033	0.409	2.5
1112872	HDP52 RM homogenate	20	0.3481	0.1538	1.0
1112873	HDP52 RC homogenate	20	0.542	0.3477	2.2
1112874	HDP53 LA homogenate	20	0.3449	0.2533	2.1
1112875	HDP53 LM homogenate	20	0.2464	0.1548	1.3
1112876	HDP53 LC homogenate	20	0.3798	0.2882	2.4
1112877	HDP53 RA homogenate	20	0.3692	0.2776	2.3
1112878	HDP53 RM homogenate	20	0.4169	0.3253	2.7
1112879	HDP53 RC homogenate	20	0.2991	0.2075	1.7
1112880	HDP54 LA homogenate	20	0.6062	0.5146	4.3
1112881	HDP54 LM homogenate	20	0.3943	0.3027	2.5
1112882	HDP54 LC homogenate	20	0.2988	0.2072	1.7
1112883	HDP54 RA homogenate	20	0.4978	0.4062	3.4
1112884	HDP54 RM homogenate	20	0.2654	0.1738	1.5
1112885	HDP54 RC homogenate	20	0.2183	0.1267	1.1
1112886	HDP55 LA homogenate	20	0.2517	0.1601	1.4
1112887	HDP55 LM homogenate	20	0.3053	0.2137	1.8
1112888	HDP55 LC homogenate	20	0.2771	0.1855	1.6
1112889	HDP55 RA homogenate	20	0.2905	0.1989	1.7
1112890	HDP55 RM homogenate	20	0.255	0.1634	1.4
1112891	HDP50 55 homogenate	20	0.2917	0.2001	1.7
1112892	HDP56 LA homogenate	20	0.3641	0.2725	2.3
1112893	HDP56 LM homogenate	20	0.3544	0.2628	2.2
1112894	HDP56 LC homogenate	20	0.3521	0.2605	2.2
1112895	HDP56 RA homogenate	20	0.398	0.3064	2.6
1112896	HDP56 RM homogenate	20	0.2339	0.1423	1.2
1112897	HDP56 RC homogenate	20	0.4484	0.3568	3.0
1112898	HDP57 LA homogenate	20	0.276	0.1844	1.6
1112899	HDP57 LM homogenate	20	0.2876	0.196	1.7
1112900	HDP57 LC homogenate	20	0.3211	0.2295	1.9
1112901	HDP57 RA homogenate	20	0.2482	0.1566	1.3

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1112902	HDP57 RM homogenate	20	0.2064	0.1148	1.0
1112903	HDP57 RC homogenate	20	0.2678	0.1762	1.5
1112904	HDP58 LA homogenate	20	0.9441	0.7715	5.8
1112905	HDP58 LM homogenate	20	0.4406	0.268	2.0
1112906	HDP58 LC homogenate	20	0.4777	0.3051	2.3
1112907	HDP58 RA homogenate	20	0.3577	0.1851	1.4
1112908	HDP58 RM homogenate	20	0.3336	0.161	1.2
1112909	HDP58 RC homogenate	20	0.4303	0.2577	2.0
1112910	HDP59 LA homogenate	20	0.3382	0.1656	1.3
1112911	HDP59 LM homogenate	20	0.4756	0.303	2.3
1112912	HDP59 LC homogenate	20	0.4498	0.2772	2.1
1112913	HDP59 RA homogenate	20	0.2655	0.0929	0.7
1112914	HDP59 RM homogenate	20	0.2563	0.0837	0.6
1112915	HDP59 RC homogenate	20	0.3454	0.1728	1.3
1112916	HDP60 LA homogenate	20	0.3106	0.138	1.1
1112917	HDP60 LM homogenate	20	0.4097	0.2371	1.8
1112918	HDP60 LC homogenate	20	0.3847	0.2121	1.6
1112919	HDP60 RA homogenate	20	0.3498	0.1772	1.3
1112920	HDP60 RM homogenate	20	0.6086	0.436	3.3
1112921	HDP60 55 homogenate	20	0.3035	0.1309	1.0
1112922	HDP61 LA homogenate	20	0.3929	0.2203	1.7
1112923	HDP61 LM homogenate	20	0.4934	0.3208	2.4
1112924	HDP61 LC homogenate	20	0.4356	0.263	2.0
1112925	HDP61 RA homogenate	20	0.418	0.2454	1.9
1112926	HDP61 RM homogenate	20	0.3637	0.1911	1.5
1112927	HDP61 RC homogenate	20	0.3771	0.2045	1.6
1112928	HDP62 LA homogenate	20	0.3578	0.1852	1.4
1112929	HDP62 LM homogenate	20	0.4016	0.229	1.7
1112930	HDP62 LC homogenate	20	0.323	0.1504	1.1
1112931	HDP62 RA homogenate	20	0.3112	0.1386	1.1
1112932	HDP62 RM homogenate	20	0.3478	0.1752	1.3
1112933	HDP62 RC homogenate	20	0.3327	0.1601	1.2

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Annex A: Procedure for Thiol and Sulfide Quantitation Kit (T-6060)**Materials****Reagents Supplied**

Papain-SSCH₃ (MW = 23,000, Component A), 18 mg,
lyophilized from buffer
N-benzoyl-L-arginine, p-nitroanilide, hydrochloride
(L-BAPNA) (MW = 434.9, Component B), 55 mg
Bis-Tris/EDTA buffer (Component C), 40 mL of 50 mM
bis-Tris, 1 mM EDTA, pH 6.3
Cystamine, dihydrochloride (MW = 225.2, Component D),
10 mg
L-Cysteine (MW = 121.2, Component E), 30 mg
Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid);
DTNB), (MW = 396.3, Component F), 10 mg

Each kit supplies sufficient material for about 50 assays using
standard 1 mL cuvettes or 250 assays using a microplate format.

Storage and Handling

Upon receipt, the kit should be stored at -20°C, protected
from light. Stored in this manner, the kit components should remain
active for at least one year. After individual components
have been made into solutions, storage at 4°C is recommended
(see Reagent Preparation, below).

Reagents Required but Not Provided

Buffer A: 5 mM sodium acetate, 50 mM NaCl, 0.5 mM EDTA, pH 4.7. Dissolve 41 mg sodium acetate and 292 mg
NaCl in about 80 mL deionized water (dH₂O). Add 1 mL of 50 mM EDTA, pH 8.0, and adjust the pH to 4.7 with 1
M HCl. Add sufficient dH₂O to bring the volume to 100 mL. Degas as indicated in Degassing.

Buffer B: 40 mM sodium phosphate, 2 mM EDTA, pH 7.6. Dissolve 0.55 g NaH₂PO₄ • H₂O in about 80 mL dH₂O.
Add 4 mL of 50 mM EDTA, pH 8.0, and adjust the pH to 7.6 with 1 M NaOH. Add sufficient dH₂O to bring the
volume to 100 mL. Degas as indicated in Degassing.

Buffer C: 5 mM sodium acetate, pH 4.0. Dissolve 41 mg sodium acetate in about 80 mL dH₂O. Adjust the pH to
4.0 with 1 M HCl. Add sufficient dH₂O to bring the volume to 100 mL. Degas as indicated in Degassing.

Protocol**Reagent Preparation**

Reagents for the Thiol and Sulfide Quantitation Kit, except for Component C, are provided as solids and must be
made into stock solutions before use. For convenience, the stock solutions of Components A, B, D and F may be
prepared directly in the component bottles, as described below. Once these solutions are prepared, they can be
stored for up to six months at 4°C. A stock solution of L-cysteine (Component E) can be prepared by weighing out
a portion of the solid and making the solution in a separate vial. L-Cysteine solutions, as thiol standards, are only
stable for about two weeks at 4°C; however, the solid is more stable, and sufficient material is provided for
preparing about six stock solutions.

1.1 Prepare a 1.2 mg/mL stock solution of papain-SSCH₃ by dissolving the 18 mg of papain-SSCH₃ (Component
A) in 15 mL of Buffer C. The final buffer composition will be approximately 5 mM sodium acetate, 50 mM NaCl,

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pH 4.5, due to salt contributions from the lyophilized material. Sodium azide, at a final concentration of 2 mM, should be added as a preservative.

1.2 Prepare a 4.9 mM stock solution of L-BAPNA by first dissolving the 55 mg of L-BAPNA (Component B) in 1.5 mL of DMSO. Sonicate briefly to help solubilize the L-BAPNA and then add 24.5 mL of bis-Tris/EDTA buffer (Component C).

1.3 Prepare a 40 mM cystamine stock solution by dissolving the 10 mg of cystamine, dihydrochloride (Component D) in 1.1 mL of Buffer A (see Reagents Required but Not Provided). Prepare a working solution of 4 mM cystamine prior to running each set of assays by diluting 100 µL of the 40 mM cystamine stock solution into 0.9 mL Buffer A.

1.4 Prepare Ellman's reagent (100 mM DTNB) by dissolving the 10 mg of DTNB (Component F) in 250 µL of DMSO.

1.5 Prepare a 100 mM L-cysteine stock solution, when needed, by weighing out 5 mg from the bottle originally containing 30 mg L-cysteine (Component E) and dissolving the sample in 412 µL of Buffer A in a separate vial.

Degassing

Important: All solutions used for thiol and sulfide determination should be degassed thoroughly before use. We recommend that solutions be degassed on ice in a vacuum desiccator at about 25 Torr for 30 minutes and then <1 Torr for an additional 30 minutes prior to use. The desiccator should be filled with argon or nitrogen gas before opening to air. Degassed solutions should be sealed and used in the assay within a short period of time. When solutions are properly degassed, the standard curve of the corrected absorbance at 410 nm vs. thiol concentration should pass through the origin; insufficient degassing will result in a standard curve that has a positive intersection on the axis of thiol concentration.

Ellman's Assay for Calibration of L-Cysteine Thiol Standard

Ellman's reagent is provided with the Thiol and Sulfide Quantitation Kit in order to accurately determine the thiol content of L-cysteine thiol standard solutions. Because thiols are prone to oxidation in aqueous solutions, the actual thiol concentration of a L-cysteine thiol standard solution may differ significantly from the nominal molarity of the solution that was prepared. The thiol concentration should be determined prior to performing assays (see Thiol and Sulfide Determination) that will be used to create a standard curve. The protocol for this assay, which uses Ellman's reagent (100 mM DTNB in DMSO; step 1.4), is presented below. For greater confidence in this determination, we recommend performing the Ellman's reagent assay in duplicate.

2.1 Dilute 5 µL of degassed 100 mM L-cysteine stock solution into 5.0 mL of degassed Buffer A to make 5.0 mL of 0.1 mM L-cysteine working solution.

2.2 Add 0.69 mL of Buffer B (see Reagents Required but Not Provided) to each of two clean tubes.

2.3 Add 0.4 mL of the 0.1 mM L-cysteine working solution to one of the tubes and 0.4 mL of Buffer A to the other, to serve as a control.

2.4 Add 10 µL of Ellman's reagent (100 mM DTNB in DMSO) to each tube, mix well and incubate the solution at room temperature for one to two minutes.

2.5 Zero the absorbance of the spectrophotometer at 412 nm using dH₂O as a blank.

2.6 Measure the absorbance of the two solutions at 412 nm. Subtract the absorbance of the control at 412 nm from the absorbance of the L-cysteine-containing solution. This is the corrected absorbance value (DA₄₁₂).

2.7 The thiol concentration of the L-cysteine working solution is calculated using the formula below:

$$\text{mM of thiol} = \frac{\Delta A_{412} \times 1.1 \text{ mL}}{13600 \times 0.4 \text{ mL}} \times 1000$$

where ΔA_{412} is the corrected absorbance value (using 1 cm-pathlength cuvettes), and 13,600 is the molar extinction coefficient (cm⁻¹M⁻¹) of the 5-thio-2-nitrobenzoate generated from Ellman's reagent in reacting with the free thiol of the L-cysteine. The concentration of the thiol determined for the 0.1 mM L-cysteine working solution will be used for generating a standard curve in the papain-SSCH₃-based assay below.

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Thiol and Sulfide Determination

The Thiol and Sulfide Quantitation Kit is designed to detect thiols in the range of $\sim 0.2 \mu\text{M}$ to $1.5 \mu\text{M}$ (~ 0.2 nanomole to 1.5 nanomoles in a 1 mL assay volume). The following step-by-step protocol describes the method for thiol determination; cysteine is used as a thiol standard. The same procedure can be used, with appropriate standards, for quantitation of inorganic sulfide, phosphines, sulfite or cyanide — for these, the determination limits are about 0.2 , 0.5 , 1 and 5 nanomoles, respectively.

3.1 Determine the number of tubes that will be needed for the standard-curve and experimental reactions. Add $15 \mu\text{L}$ of the 4 mM cystamine working solution (step 1.3) to each tube. Cystamine is required as an intermediate disulfide for the determination of protein thiols (sulfhydryls) and for the determination of certain thiols that have high pK_a values.

3.2 To the standard-curve reaction tubes, add 0 , 2 , 5 , 8 , 12 and $15 \mu\text{L}$ of 0.1 mM L-cysteine working solution (step 2.1). Add sufficient Buffer A to make the volume added in this step equal $15 \mu\text{L}$; the total volume in each tube will now be $30 \mu\text{L}$.

3.3 To the tubes for the experimental samples, add $15 \mu\text{L}$ sample volumes, prepared in Buffer A. The experimental samples added should contain 0.2 nanomole to 1.5 nanomoles of the thiol or inorganic sulfide; a dilution series may be required to attain this target range.

3.4 Prepare a 0.6 mg/mL papain-SSCH₃ working solution by mixing equal volumes of 1.2 mg/mL papain-SSCH₃ stock solution (step 1.1) and Buffer B (see Reagents Needed but Not Provided).

A 0.5 mL volume will be used in each reaction; prepare a slight excess. This working solution of papain-SSCH₃ should be freshly prepared before each set of assays; between experiments, the papain-SSCH₃ is best stored as the 1.2 mg/mL stock solution.

3.5 Add 0.5 mL of the 0.6 mg/mL papain-SSCH₃ working solution to each assay tube and mix well.

3.6 Incubate the reactions at room temperature for about one hour.

3.7 Add 0.5 mL of the 4.9 mM L-BAPNA solution (step 1.2) to each assay tube and mix well. For consistent incubation periods (see below), the L-BAPNA substrate can be added to the individual tubes, with starting times offset by one minute, for example.

3.8 Incubate the reactions at room temperature for one hour. The exact time interval is not critical; however, it is essential that all reactions be incubated for the same length of time.

3.9 Zero the absorbance of the spectrophotometer at 410 nm using dH_2O as a blank, and measure the absorbance at 410 nm of each reaction. For accuracy, the absorbance value must be between 0.1 and 1 . Dilution of the sample may be necessary.

3.10 Calculate the corrected absorbance by subtracting the absorbance value for the control reaction lacking L-cysteine from absorbance values for both the standard reactions and experimental reactions. For the samples that required dilution, multiply the corrected value by the dilution factor.

3.11 Plot a curve of the corrected absorbance at 410 nm of the L-cysteine standards vs. the calibrated thiol content of these samples as determined from results of the Ellman's assay.

3.12 Read the thiol (or sulfide) concentration of experimental samples from the standard curve.

END OF REPORT

Dstl/DTD/UKAS/Form 3 Issue 08 September 2008

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UNCLASSIFIED

ANNEX D

Data for the analysis of desmosine in urine and terminal BAL, and MMP, neutrophil elastase and myeloperoxidase in terminal BAL, in pigs following sulphur mustard exposure with and without post-treatment with N-acetyl cysteine.

Janis Shute, Reader in Pharmacology, University of Portsmouth, 14th February 2012.

Methods:

Animals were randomly assigned to either HD control (100 µg.kg⁻¹ HD; n=6) or HD treated (100 µg.kg⁻¹ HD followed by NAC (1 ml of 200 mg.ml⁻¹ solution of Mucomyst™ at + 30 min, 2, 4, 6, 8 and 10 hours post exposure; estimated total dose delivered 1200 mg; n=8)) groups. NAC was administered as an aerosol generated by an Aeroneb® Lab micropump nebuliser (Aerogen (Ireland) Ltd, Galway Ireland). The nebuliser was run until dry (approx 5 min). Control animals had the nebuliser and spacer attached for 5 min, but the nebuliser was not switched on.

BAL samples were received from 5 untreated animals and 8 NAC-treated animals and stored at -80°C. Urine was collected at 30 minutes and every hour post sulphur mustard exposure for 12 hours and stored frozen (-80°C).

Desmosine was measured by specific ELISA (Protocol 1 attached), using neat samples of urine and BAL fluid.

Matrix metalloproteinase (MMP) activity was measured using the Enzcheck assay kit (Invitrogen) according to the manufacturer's instructions on neat BAL, over 3 hours.

Neutrophil elastase (NE) activity was measured using an in-house assay (Protocol 2 attached) on neat BAL fluid, over 21 hours.

Myeloperoxidase (MPO) activity was measured using an in-house assay (Protocol 3 attached) over 1 hour.

Results;

Individual animal variation in urinary desmosine production in animals exposed to HD is shown in Figure 1a. A significant increase ($p = 0.03$) in urinary desmosine was noted at 4 h post HD exposure, which seemed to decline at 7 - 8 h and then increase again (Figure 1b). In a previous study (Dstl/TR50669, Annex A) we similarly reported a significant increase in BAL desmosine 4 h post HD exposure, but the significantly higher levels were maintained at 6,8, and 10 h.

Individual animal variation in urinary desmosine production in animals exposed to HD + NAC is shown in Figure 1c, mean data \pm SEM for all animals is shown in figure 1d.

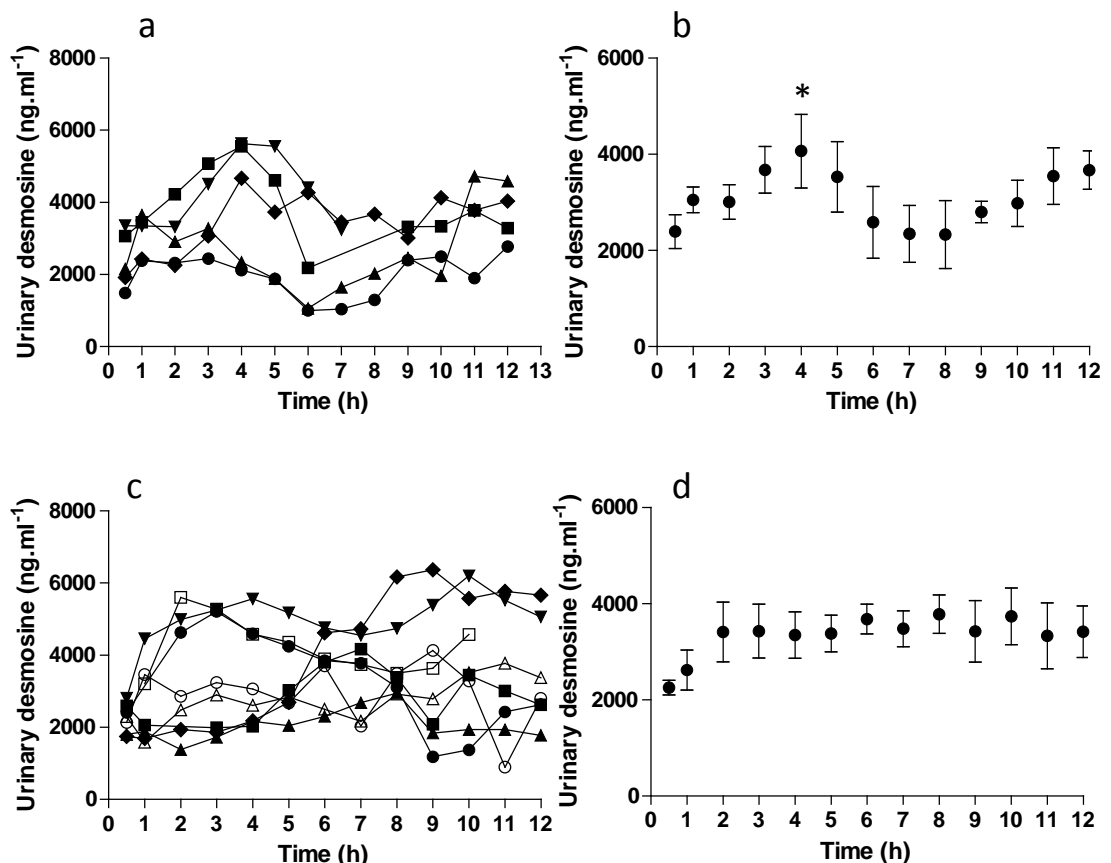


Figure 1: Urinary desmosine production. a) Individual animals from HD group, b) HD group, mean \pm SEM, c) Individual animals from HD + NAC group and d) HD + NAC group, mean \pm SEM. (*= $p < 0.05$).

Because some urine samples were missing in some sets, a one-way Anova was not possible, therefore only the data sets ($n=5$) with all samples present were used for statistical analysis (below) that shows that NAC has ablated the early increase in urinary desmosine, but that there seems to be a delayed increase in desmosine levels that was significant 8, 9 and 10 h post HD exposure (figure 2a). No significant difference could be detected by simple 2-way ANOVA (not repeat measures ANOVA) which allows for missing data points between HD and HD + NAC animals (Figure 2b)

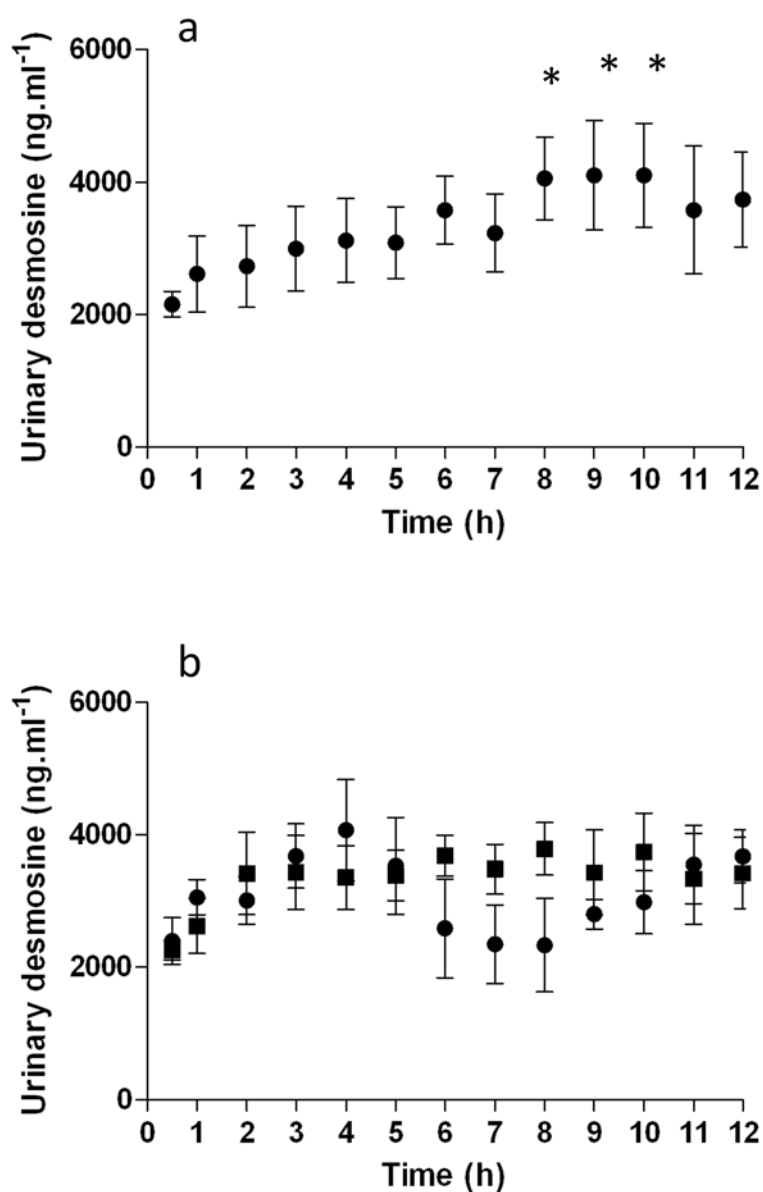


Figure 2: Urinary desmosine production. a) Complete data sets from HD + NAC group mean \pm SEM (n=5), b) comparison of HD (●) vs HD + NAC (■) groups (mean \pm SEM). (*p < 0.05).

There was no significant difference in terminal BAL fluid desmosine concentration between HD control and NAC treated groups (Figure 3).

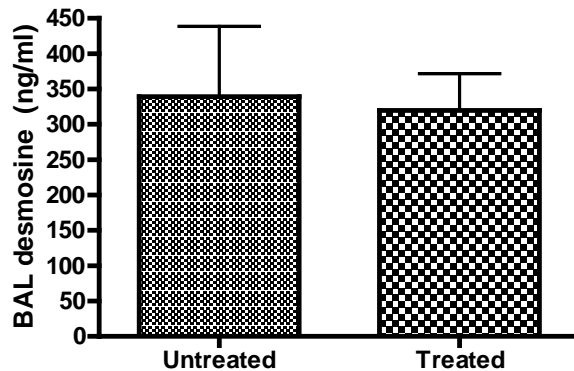


Figure 3: Terminal BAL fluid desmosine concentration in HD controls and NAC treated animals. Mean \pm SEM.

There was no significant difference in terminal BAL fluid MPO activity data between HD control and NAC treated groups, although MPO activity in the NAC-treated animals was lower than HD controls, this was not significant (Figure 4; $p = 0.08$).

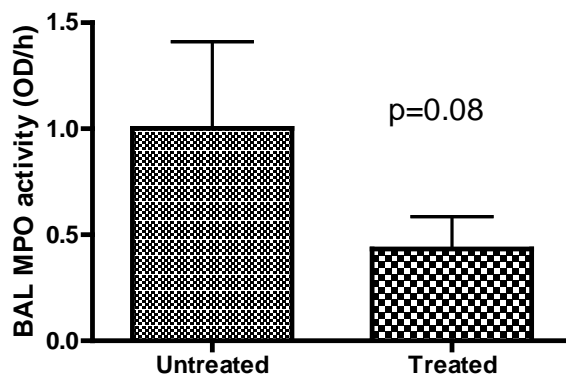


Figure 4: Terminal BAL fluid myeloperoxidase (MPO) activity in HD controls and NAC treated animals. Mean \pm SEM.

There was no significant difference in terminal BAL fluid NE activity between HD control and NAC treated groups (Figure 5). Again, NE activity was lower in the treated animals but not significantly so (NS).

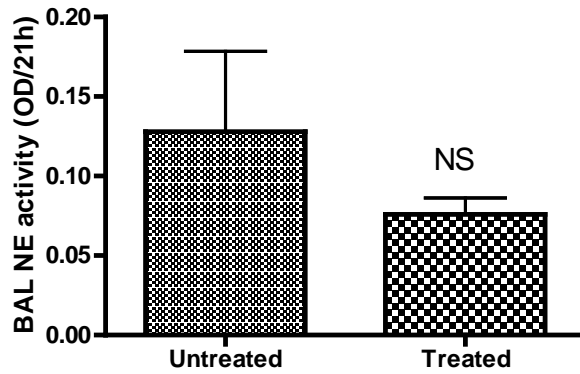


Figure 5: Terminal BAL fluid neutrophil elastase (NE) activity in HD controls and NAC treated animals. Mean \pm SEM.

There was no significant difference in terminal BAL fluid MMP activity (measured as gelatinase) between HD control and NAC treated groups (Figure 6). Again, MMP activity was lower in the treated animals but not significantly so ($p = 0.1$).

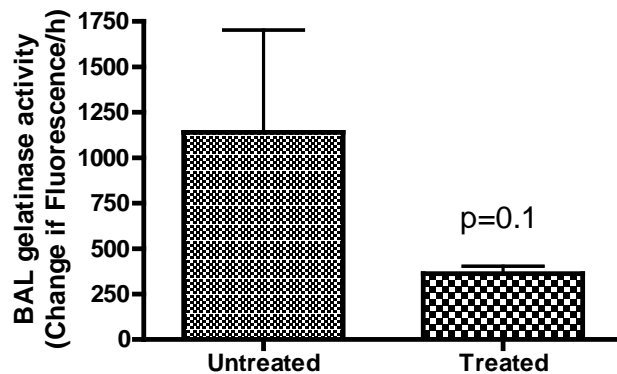


Figure 6: Terminal BAL fluid matrix metalloproteinase (MMP) activity in HD controls and NAC treated animals. Mean \pm SEM.

Terminal lavage fluid differential WBC counts were provided by Dstl, Porton Down, showing that the % neutrophils in the BAL were significantly reduced following NAC treatment compared with HD controls (Figure 7; $p = 0.015$). However, we could find no significant correlation between neutrophil number and any other parameter measured.

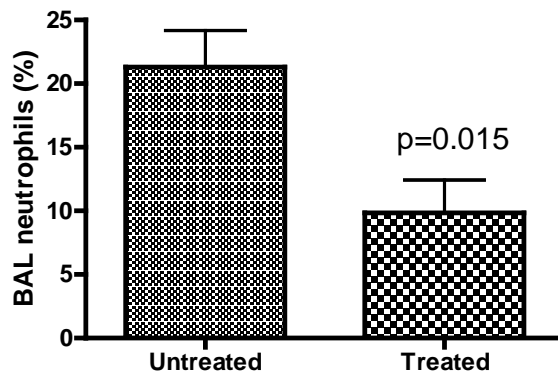


Figure 7: Terminal BAL fluid neutrophil count in HD controls and NAC treated animals. Mean \pm SEM.

Conclusions;

NAC significantly reduced the inflammatory response when measured as the recruitment of neutrophils into the airways, and delayed the increase in urinary desmosine concentration from 4 h post exposure to 8 h. However, by 12 h there was no significant difference in urine or BAL fluid desmosine and the effect of NAC on elastin degradation appears to have been transient. The decrease in the percentage of neutrophils in the BAL fluid at 12 h was mirrored by a decrease in the level of neutrophil elastase activity, MPO and MMP activity, although significant effects were not seen.

Most previous studies report an anti-inflammatory and anti-oxidative effect of NAC. However, NAC is generally administered prior to HD exposure, by the oral or intravenous route. In studies where NAC was given post HD-exposure it was via the intravenous route and reduced lung injury, in the rat.

Reference; Weinberger B et al. Sulfur mustard-induced pulmonary injury: therapeutic approaches to mitigating toxicity. *Pulm Pharmacol Ther* 2011; 24 (1): 92-99.

A competitive ELISA that detects urinary desmosine (and isodesmosine)

Assay-specific reagents were purchased from;
Elastin Products Company (EPC)
PO Box 568
Owensville
Missouri 65066 , USA
www.elastin.com
email; epc@fidnet.com
Phone; (573) 437-2193
FAX; (573) 437-4632

The assay was developed from the following key references;

1. Laurent P *et al.* Quantitation of elastin in human urine and rat pleural mesothelial cell matrix by a sensitive avidin-biotin ELISA for desmosine. J Immunol Methods 1988; 107: 1-11.
2. Osakabe T *et al.* Comparison of ELISA and HPLC for the determination of desmosine or isodesmosine in aortic tissue elastin. J Clin Lab Analysis 1995; 9: 293-296.
3. Cumiskey WR *et al.* Enrichment and analysis of desmosine and isodesmosine in biological fluids. J Chromatog B 1995; 668: 199-207.

Reagents;

1. Desmosine–egg albumin (DEA) conjugate (EPC # DA855)
2. Desmosine standard (EPC # D866)
3. Isodesmosine standard (EPC # D975)
4. Rabbit anti-desmosine serum (EPC # DA878).
This antibody has no species specificity but we found it cross reacted with isodesmosine.

Buffers;

A1; (veronal buffer-VBS) 0.01M veronal (barbitone), 0.15M NaCl, 1 mM MgCl₂ and 1 mM CaCl₂.
To 100 ml UHQ water add 0.1842 g veronal, 0.8766 g NaCl, 0.0203 g MgCl₂.6H₂O, 0.0147 g CaCl₂.2H₂O, and adjust pH to 7.2 with 1M NaOH.

A2; To buffer A1 add 0.1% V/V Tween 20 and 0.1% W/V pig serum albumin (or 0.1% V/V pig serum).

B; 0.1M TRIS-HCl, 0.1% V/V Tween-20 and 0.1% W/V pig albumin (or 0.1% V/V pig serum).
To 100 ml UHQ water add 1.211 g TRIS base and adjust pH to 7.2 with conc HCl and add Tween 20 (0.1 ml) and pig serum albumin (0.1 g).

Method;

1. DEA (5 mg) was dissolved in 0.5 ml water to give a 10 mg/ml stock solution and stored in plastic Eppendorf tubes (avoid glass) at -20C (although -80C might be better as we found the sensitivity of the assay declined over time). The DEA was diluted 1:4000 to 2.5 µg/ml in bicarbonate buffer pH 9.6 (Sigma capsules dissolved in water). The wells of a 96-well microtitre plate were

coated with 250 ng DEA in 100 µl bicarbonate buffer overnight at 4C in the dark.

2. Desmosine and isodesmosine standards (5 mg) were dissolved in 0.5 ml Buffer B, aliquoted and stored at -20C (10 mg/ml stock). On the day of the assay, the stock was diluted 1:1000 in Buffer B (10 µg/ml) then standards were prepared in the range 0, 5, 10, 50, 100, 500, 1000, 2000 ng/ml Buffer B.
3. Urine samples (early morning 'spot' mid-stream) were diluted 1:10 with UHQ water. (Hydrolysis of 6 urine samples in a final concentration of 6M HCl (ref 3), vacuum evaporation, redissolving in UHQ water and centrifugation (10 min 1500 g) to remove insoluble material, produced non-significantly(p=0.075) higher levels of desmosine but this needs to be confirmed. There are conflicting previous reports on recovery of desmosine in urine)
4. Rabbit anti-desmosine serum was prepared at a dilution of 1:3000 in Buffer B.
5. Standards and samples (100 µl) were added to 200 µl of rabbit anti-desmosine serum and 50µl Buffer B and incubated at 37C for 30 min, then placed on ice to cool.
6. Plates were washed 6X with 150 µl PBS/0.1% Tween-20.
7. Standards and samples (100 µl in duplicate) were added to the wells of the microtitre plate and the plate covered and incubated for 2h at 4C.
8. Plates were washed 6x with 150 µl PBS/Tween.
9. Biotinylated swine anti-rabbit (Dako) was diluted 1:1000 in Buffer A2 and 100 µl added to each well and left to incubate for 1h at room temperature.
10. SAB complexes (Dako) were prepared at 1:100 dilution (1µl A plus 1µl B in 100 µl Buffer A2) for 30 min at room temperature, then diluted to 10 ml in buffer A2 for use.
11. Plates were washed 6X as before and 100 µl SAB complexes added to each well and left for 30 min at room temperature.
12. Substrate (5.5 mM o-phenylene-diamine solution in TRIS-citrate buffer pH 6) was prepared;

To 50 ml UHQ water add 1.21 g TRIS, 0.6 g citrate, 50 ul 30% H₂O₂, 0.03 g o-phenylene diamine.
13. Plates were washed 6X and 100 ul of the substrate solution added to each well and colour allowed to develop for 30 min at 37C.
14. Reactions were stopped by the addition of 100 ul 2M H₂SO₄ and the plate read at 490 nm.

BALF myeloperoxidase assay

1. OPD substrate;

0.09g o-phenylene diamine dissolved in 50 ml of 50mM phosphate-citrate buffer, and add 4ul of 30% H_2O_2 to 50 ml substrate solution.

2. Phosphate-citrate buffer

i) 50 mM sodium phosphate Na_2HPO_4 ; 0.71g in 100 ml water

ii) 50mM citric acid; 0.961g in 100 ml water.

Adjust the pH of sodium phosphate solution with the citric acid solution to pH 5.0.

3. Assay;

Incubate 100 ul of sample with 100 ul substrate in phosphate-citrate buffer.

Incubate at 37°C until a strong colour develops.

The reaction was stopped by the addition of 50 μ l 2M Sulphuric acid (H_2SO_4) and the plate read at 490nm.

Protocol 3.

NE activity

NE activity was measured in BAL samples that had been thawed once only.

BALF samples (10µl) were placed in the wells of a 96-well microtitre plate, and pre-incubated for one minute at 37°C. Then 90µl substrate (0.555mM N-methoxysuccinyl-ala-ala-pro-val-p-nitroanilide (Sigma, UK) in assay buffer) was added and the plate incubated for ten minutes at 37°C. The colour change was read as an increase in absorbance at 410nm using a microtitre plate reader (Dynatech, UK).

Assay buffer (0.3M TRIS-HCl, containing 1.5M NaCl, pH 8.0).

0.3M TRIS base – 0.36 g/10ml

1.5M NaCl – 0.87 g/10ml

Add conc HCl to pH 8.0, H₂O to 10ml.

Substrate;

N-methoxysuccinyl-ala-ala-pro-val-p-nitroanilide is made up as a concentrated stock solution in dimethylsulphoxide (DMSO) at 88.9 mM (ie 10 mg/190µl) and stored in 20µl aliquots at –20C. On the day of assay, aliquots were diluted to 0.555mM in assay buffer;

ie each 20 µl aliquot was diluted to 3.2ml in assay buffer, as required.

ANNEX E Measured parameters

This annex provides a detailed list of all of the parameters that were measured and recorded as part of the study. It also includes all of the data (in graphical form) collected during the study, for those parameters that were not included in the main body of the report. No statistical analysis of the data presented here has been performed.

A Glossary of the measured parameters is shown below the table.

	Parameters
Cardiac physiology (PiCCO)	Mean arterial pressure SVR Heart rate Continuous Cardiac output Stroke Volume Stroke Volume Variation Pulse Pressure Variation dP Max
PiCCO (thermodilution)	Cardiac output CFI GEDV ITBV Extra Vascular Lung Water PVPI GEF
Animal monitoring (Propaq)	Core Temperature Respiratory rate Heart rate Expired CO ₂ Pulse oximetry Mean arterial pressure Central venous pressure
Blood Chemistry (arterial and venous)	pH pO ₂ pCO ₂ Sodium Potassium Calcium Glucose Lactate Haematocrit Temperature Haemoglobin Oxygen saturation (SO ₂) Oxyhaemoglobin Carboxyhaemoglobin Met Haemoglobin HHb RHb pH temperature corrected pO ₂ temperature corrected pCO ₂ temperature corrected Calcium corrected to pH 7.4 HCO ₃

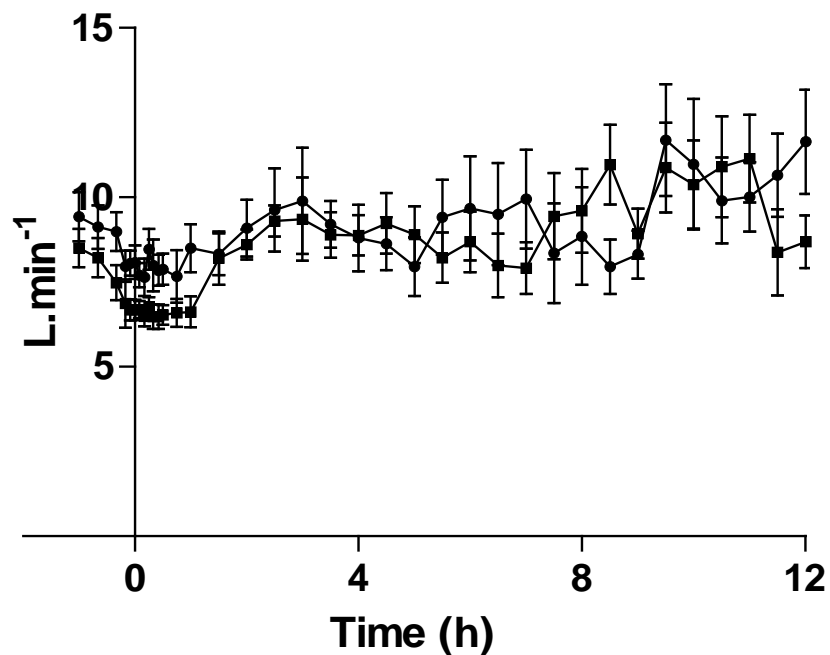
	$\text{HCO}_3^{\text{std}}$ TCO_2 Base excess O_2^{ct}
Derived cardiovascular variables	Left ventricular stroke work (indexed)
Derived O_2 transport variables	Oxygen delivery and consumption Alveolar O_2 content Mixed venous O_2 content Shunt fraction
Post experiment analysis	Whole blood <ul style="list-style-type: none"> – Haematology – Blood smears Plasma <ul style="list-style-type: none"> – ELISAs (IL-1β, IL-6, CRP, LTB₄, Thromboxane B₂, TNF-α) – Clinical chemistry (parameters listed in Annex B) Lavage <ul style="list-style-type: none"> – Protein concentration – Cell differentials – ELISA (IL-1β, IL-6, IL-8, LTB₄, Thromboxane B₂, TNF-α) Urine <ul style="list-style-type: none"> – Desmosine analysis – MSMTESE – Volume – pH Tissue <ul style="list-style-type: none"> – Genomics – LWW:DW ratio – LWW:BW ratio

Cardiovascular Parameters

– CFI	– Cardiac Function Index
– CO	– Cardiac Output
– CvO ₂	– Mixed Venous Oxygenation
– CVP	– Central Venous Pressure
– DO ₂	– Oxygen Delivery
– dpMax	– Maximum diastolic pressure
– EVLW	– Extravascular Lung Water
– GEDV	– Global End Diastolic Volume
– GEF	– Global Ejection Fraction
– ITBV	– Intra Thoracic Blood Volume
– LVSW	– Left Ventricular Stroke Work
– LWW:BW	– Lung Wet Weight to Body Weight ratio
– LWW:DW	– Lung Wet Weight to Dry Weight ratio
– MAP	– Mean Arterial Pressure
– PAO ₂	– Alveolar Oxygen Concentration
– PiCCO & CO	– Pulse Contour Cardiac Output
– PPV	– Pulse Pressure Variation
– PVPI	– Pulmonary Vascular Permeability Index
– Qs:Qt	– Shunt fraction
– SV	– Stroke Volume
– SVR	– Systemic Vascular Resistance
– SVV	– Stroke Volume Variation
– V O ₂	– Oxygen Consumption

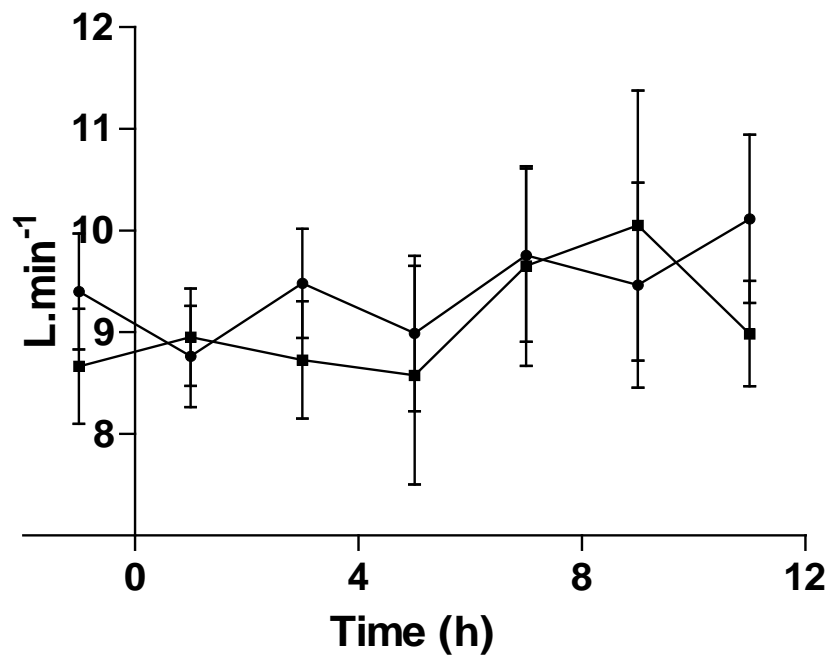
Parameters measured by PiCCO

PiCCO



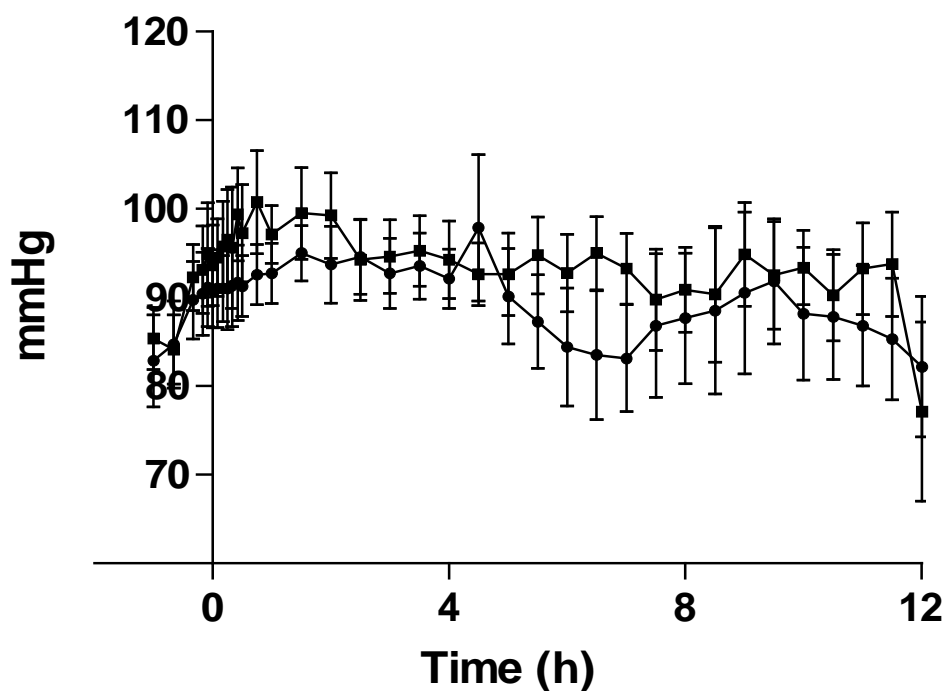
Changes in continuous cardiac output following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

CO



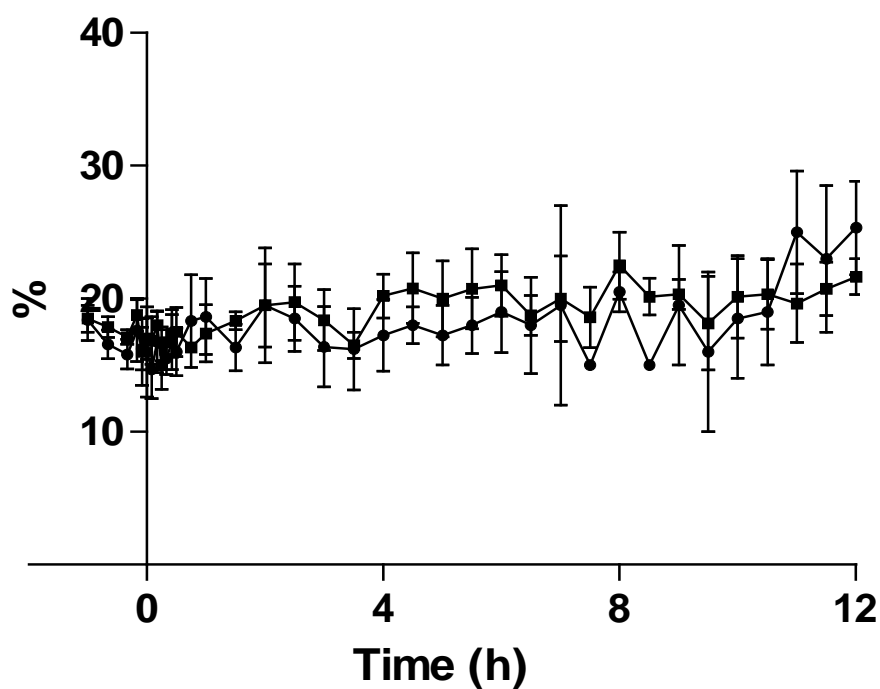
Changes in cardiac output following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

MAP



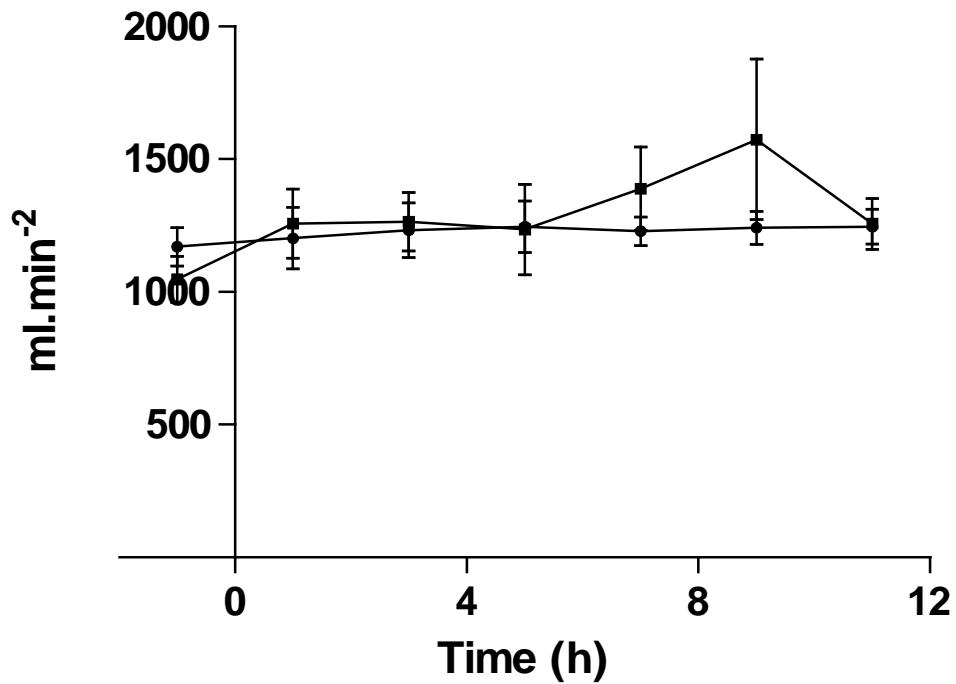
Changes in mean arterial pressure following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

PPV



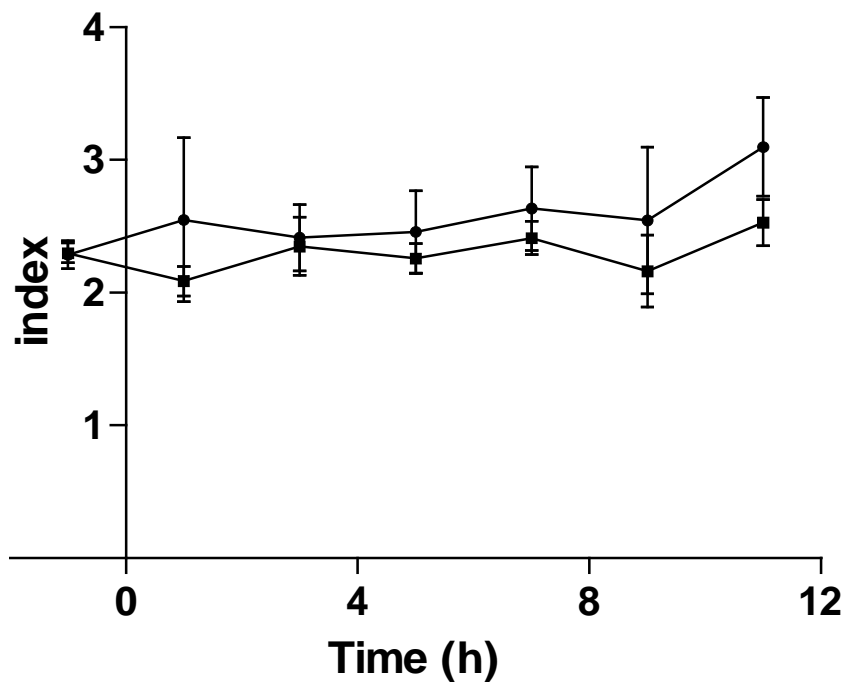
Changes in pulse pressure variation following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

ITBV



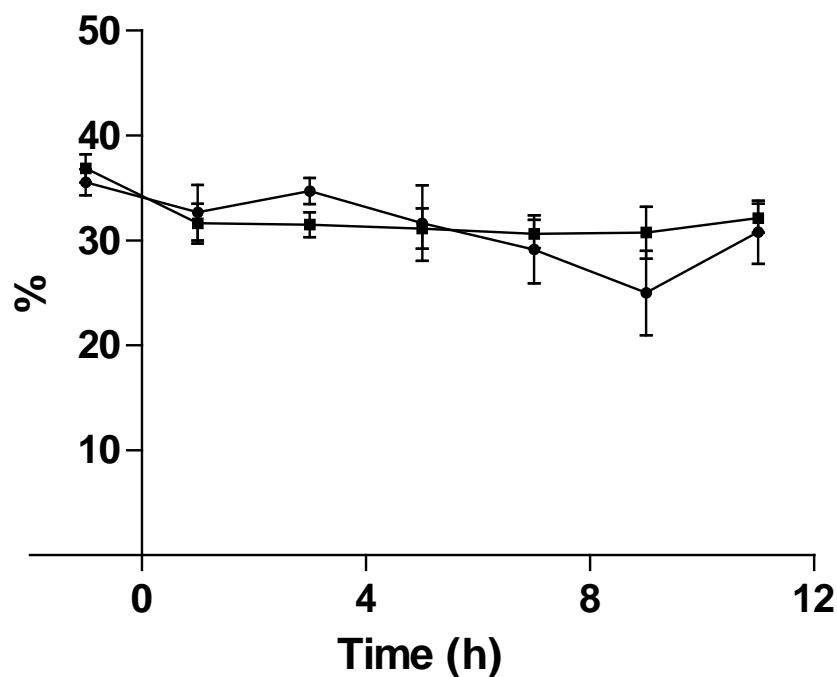
Changes in intrathoracic blood volumes following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

PVPI



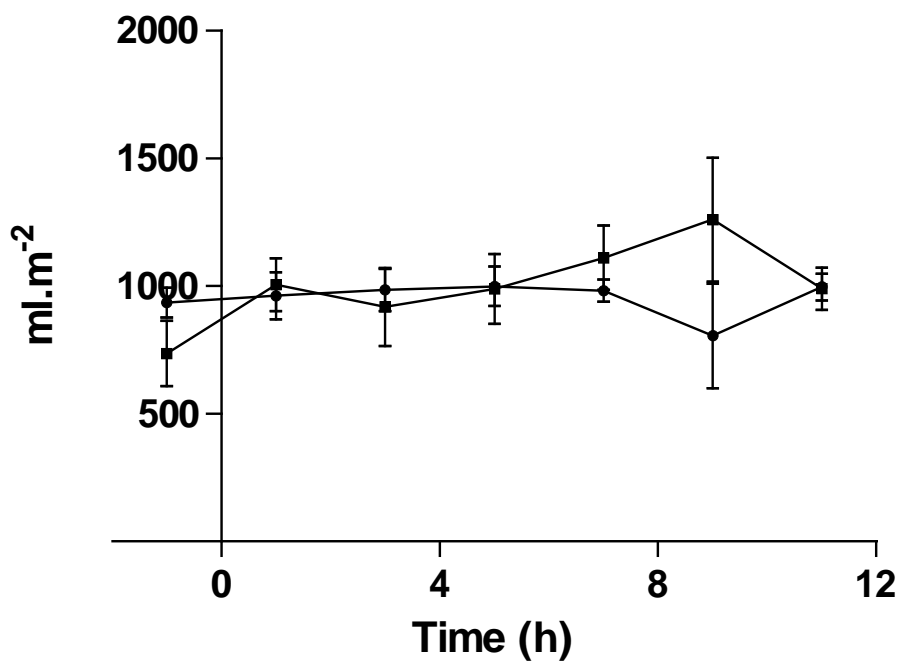
Changes in pulse pressure variation index following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

GEF

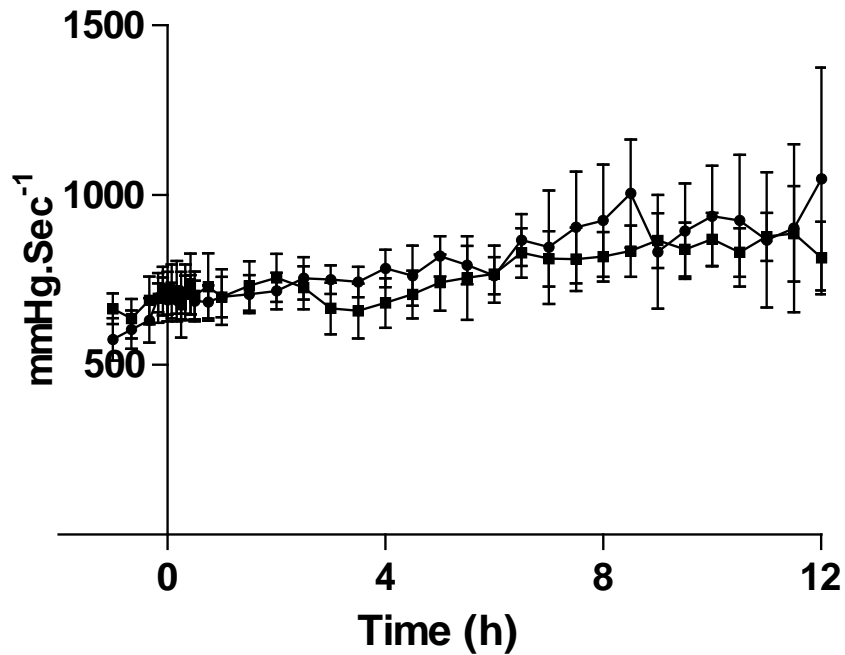


Changes in global end fraction following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

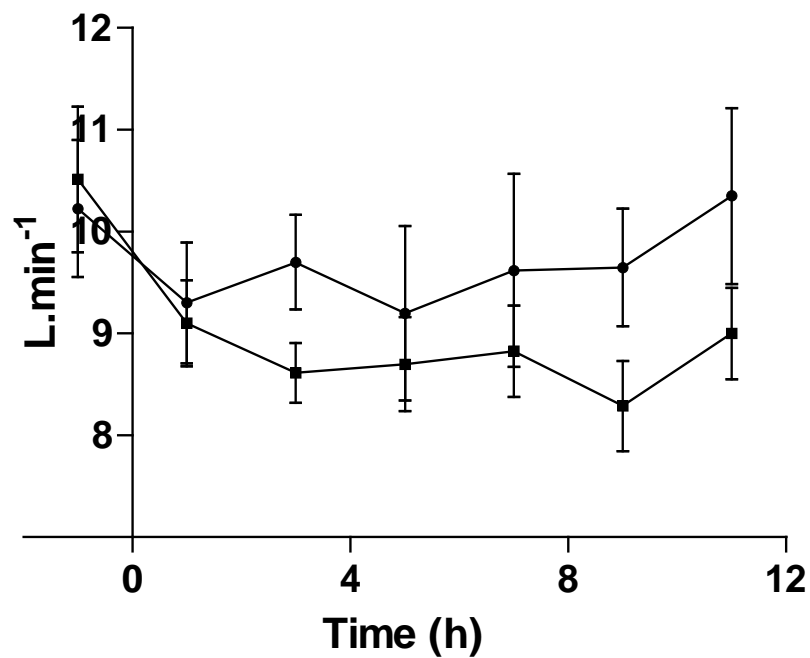
GEDV



Changes in global end diastolic volumes following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

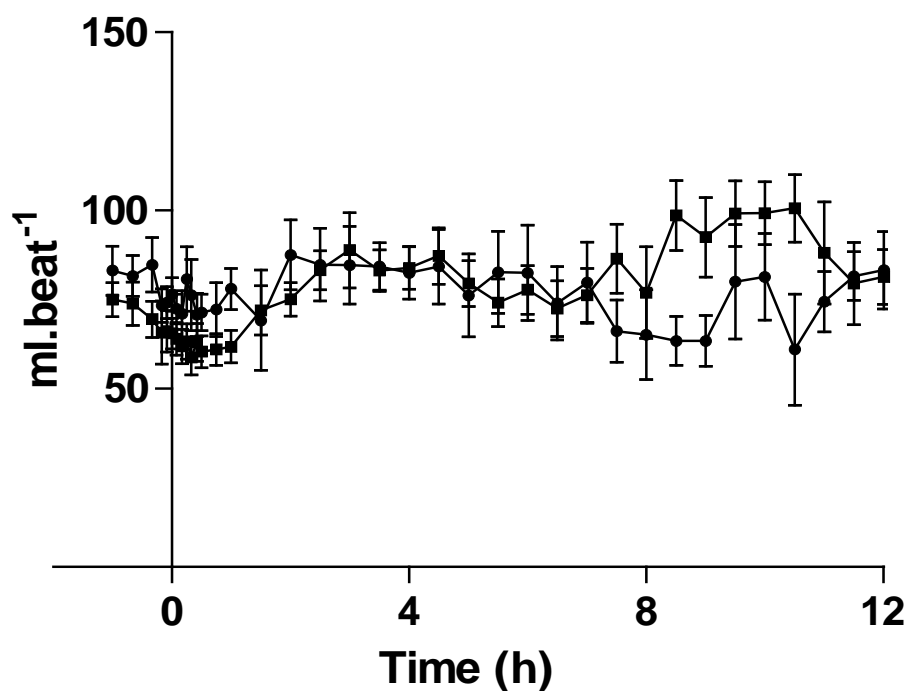
dpMax

Changes in maximum diastolic pressure following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

CFI

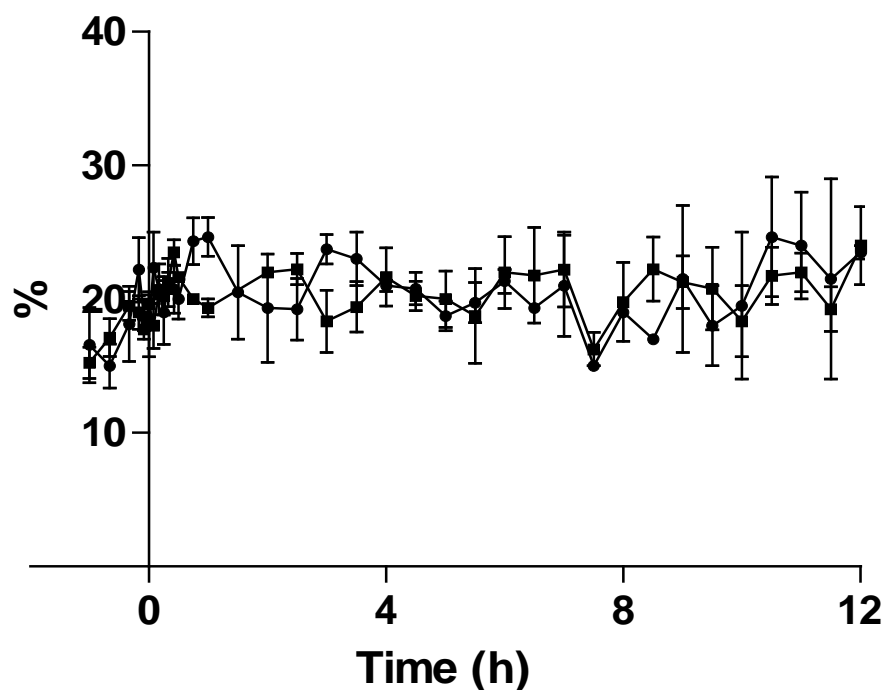
Changes in cardiac function index following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

SV



Changes in stroke volume following exposure to HD: HD controls (●) and HD + NAC (■). Data are expressed as mean \pm SEM.

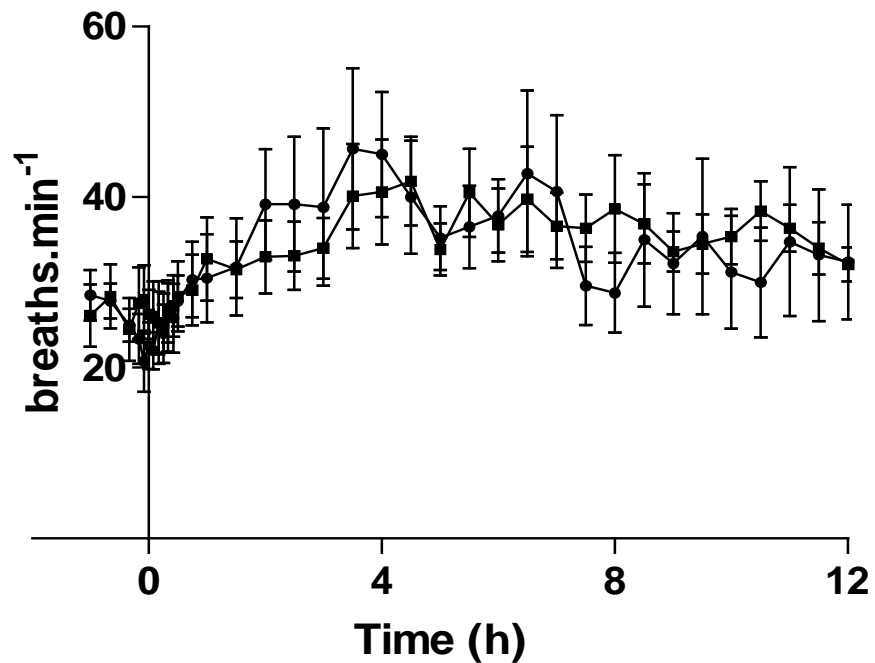
SVV



Changes in stroke volume variation following exposure to HD: HD controls (●) and HD + NAC (■). Data are expressed as mean \pm SEM.

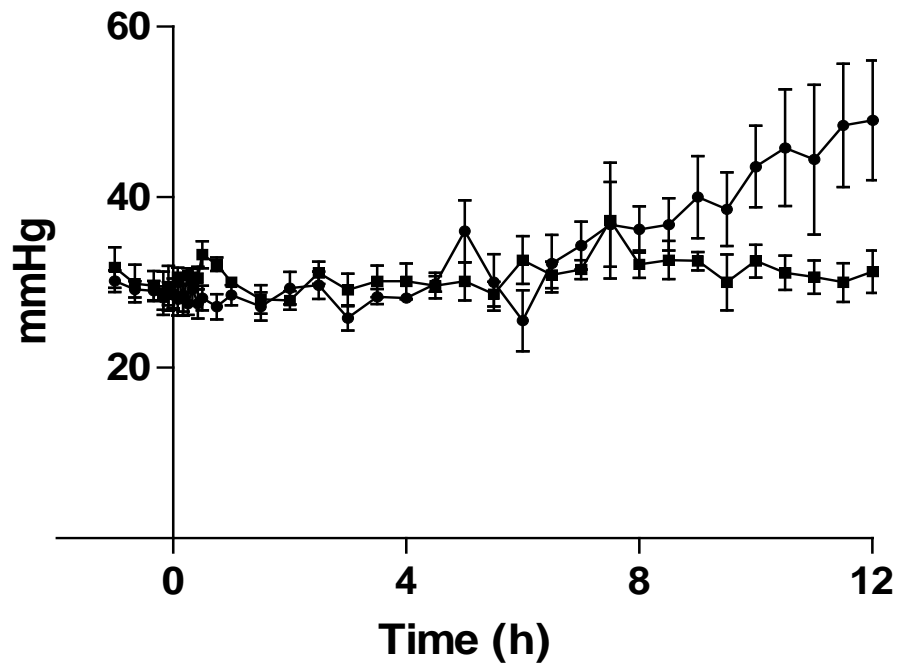
Parameters measured by Propaq

Respiration Rate



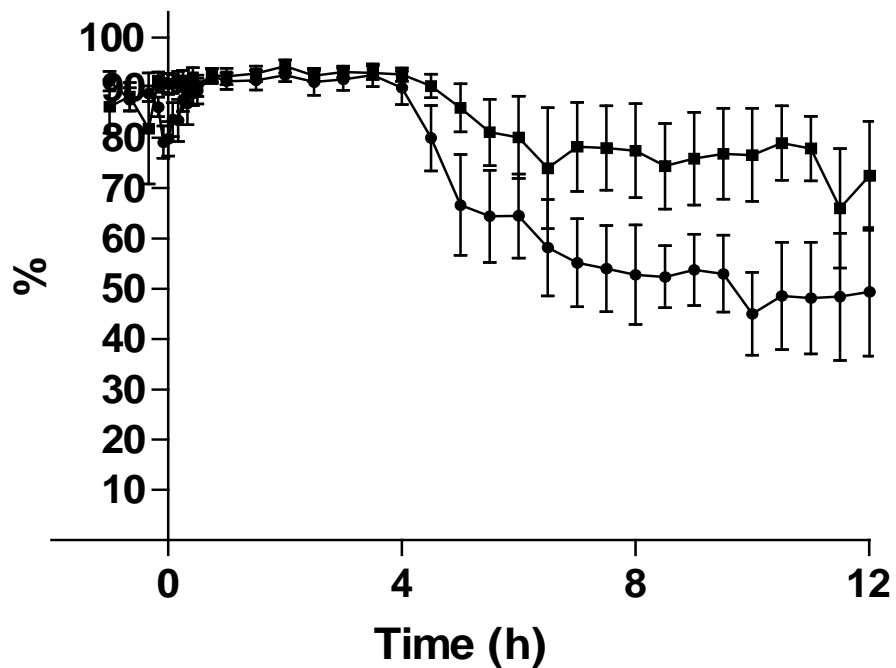
Changes in respiration rate following exposure to HD: HD controls (●) and HD + NAC (■). Data are expressed as mean \pm SEM.

Pulse Pressure



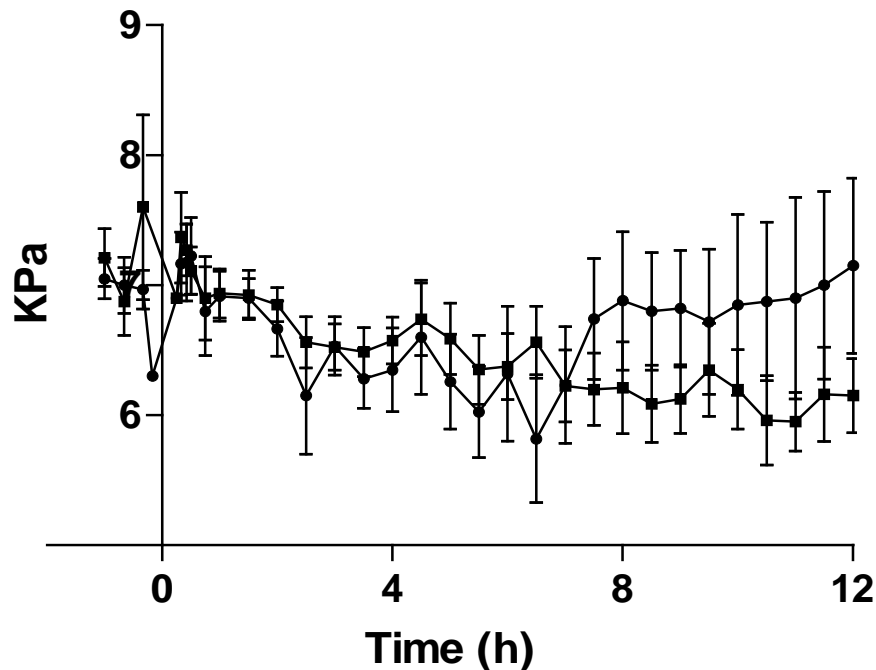
Changes in pulse pressure following exposure to HD: HD controls (●) and HD + NAC (■). Data are expressed as mean \pm SEM.

Pulse Oximetry



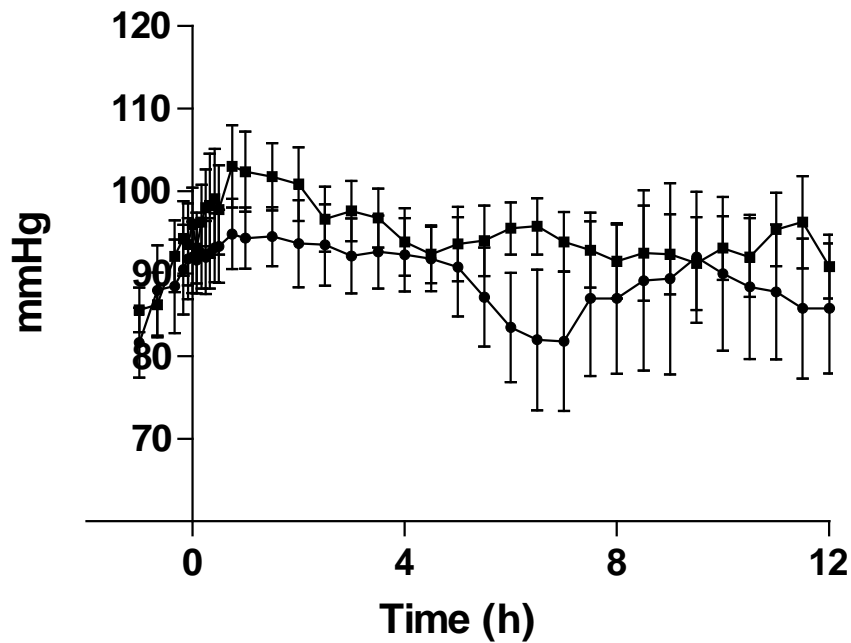
Changes in pulse oximetry following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

Expired CO₂



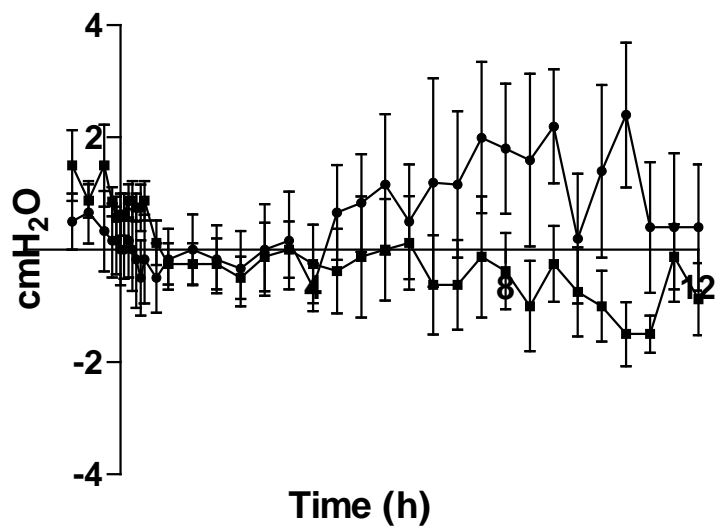
Changes in expired carbon dioxide following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

MAP



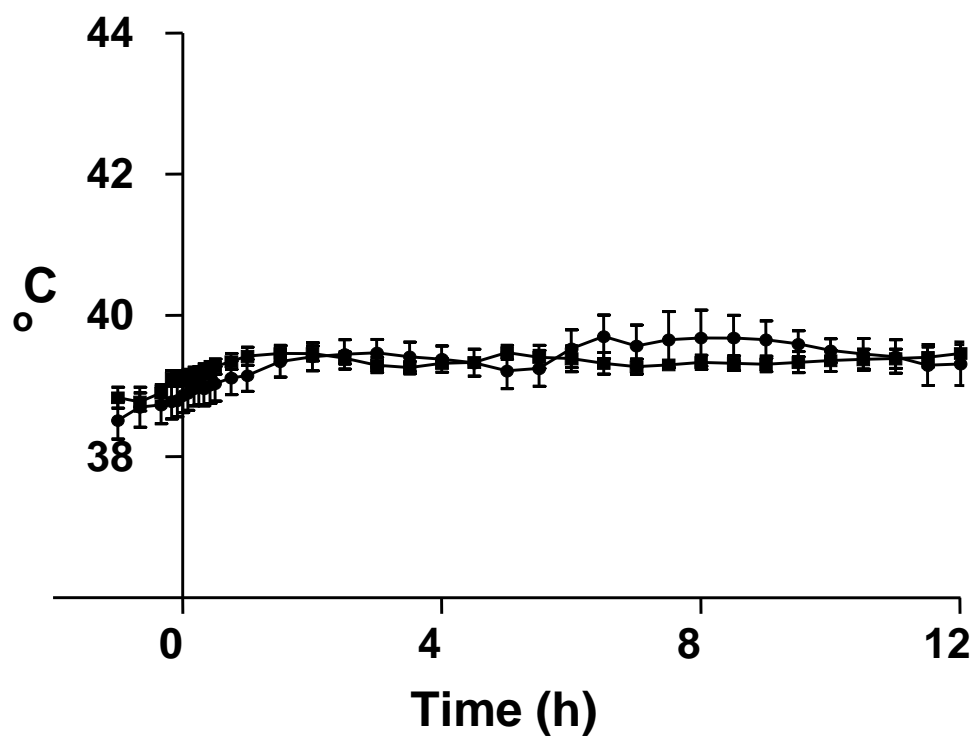
Changes in mean arterial pressure following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

CVP



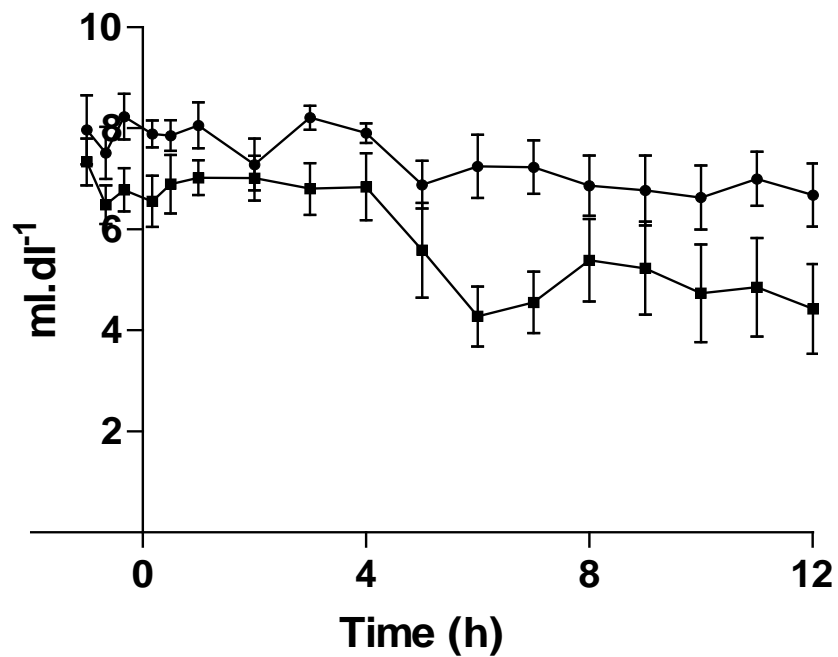
Changes in central venous pressure following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

Core Temperature

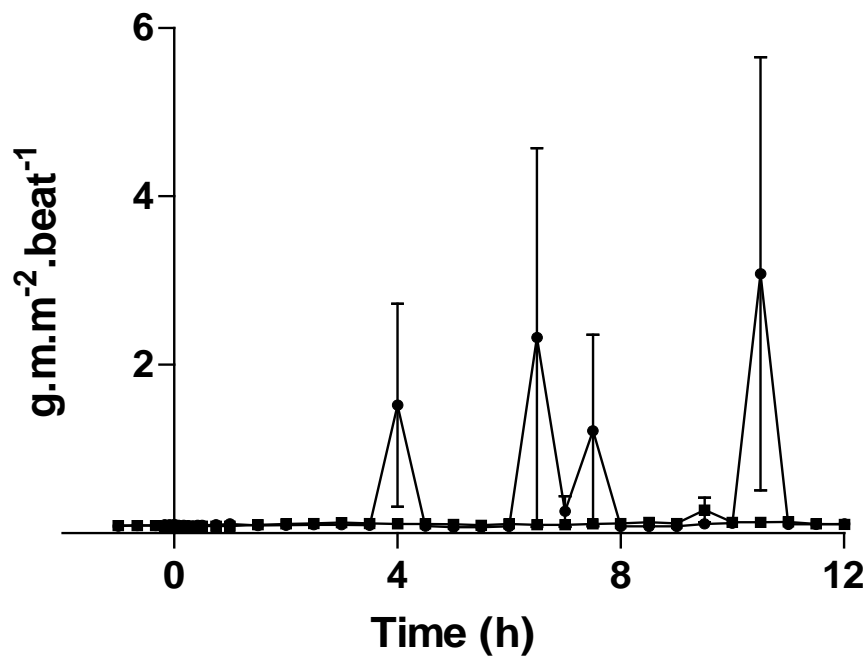


Changes in core temperature following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

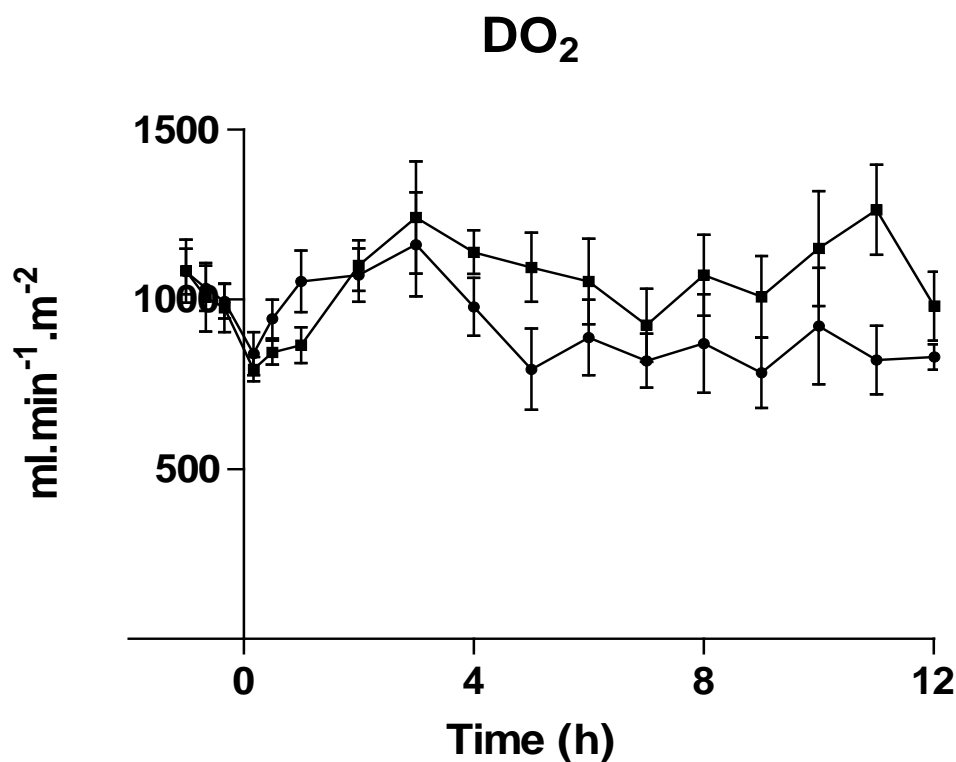
Derived Parameters

CvO₂

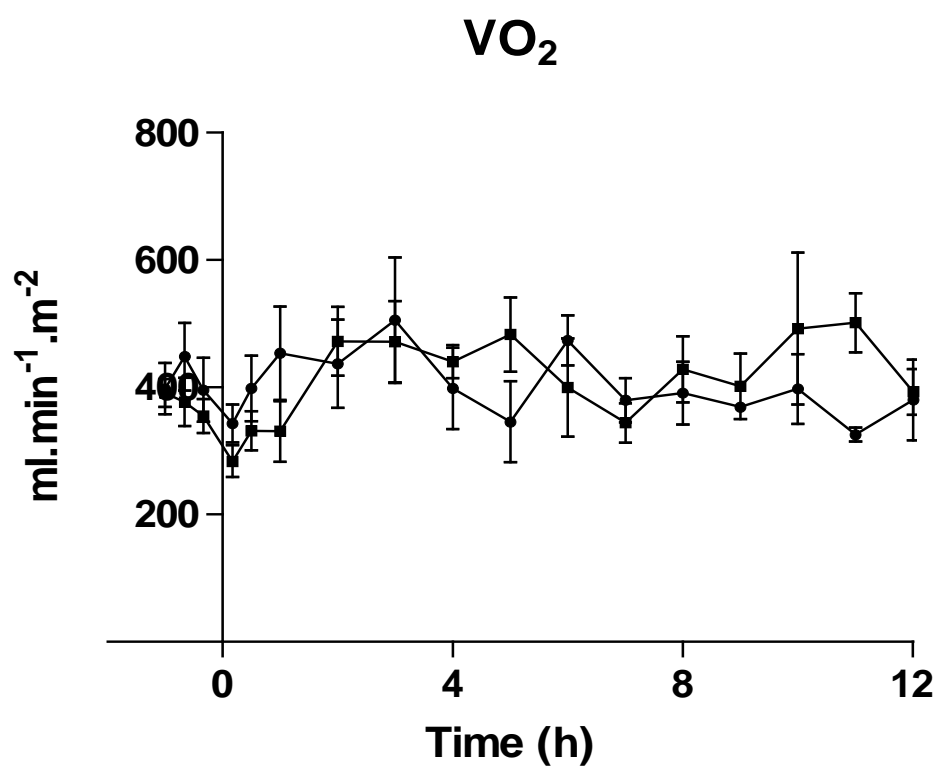
Changes in mixed venous oxygenation following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

LVSW

Changes in left ventricular stroke work following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.



Changes in oxygen delivery following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

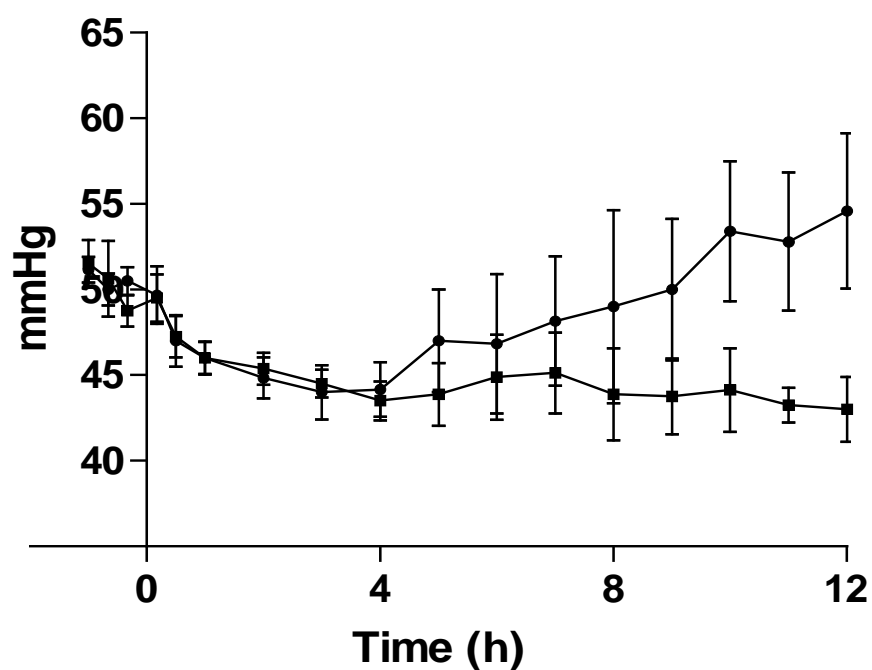


Changes in oxygen consumption following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

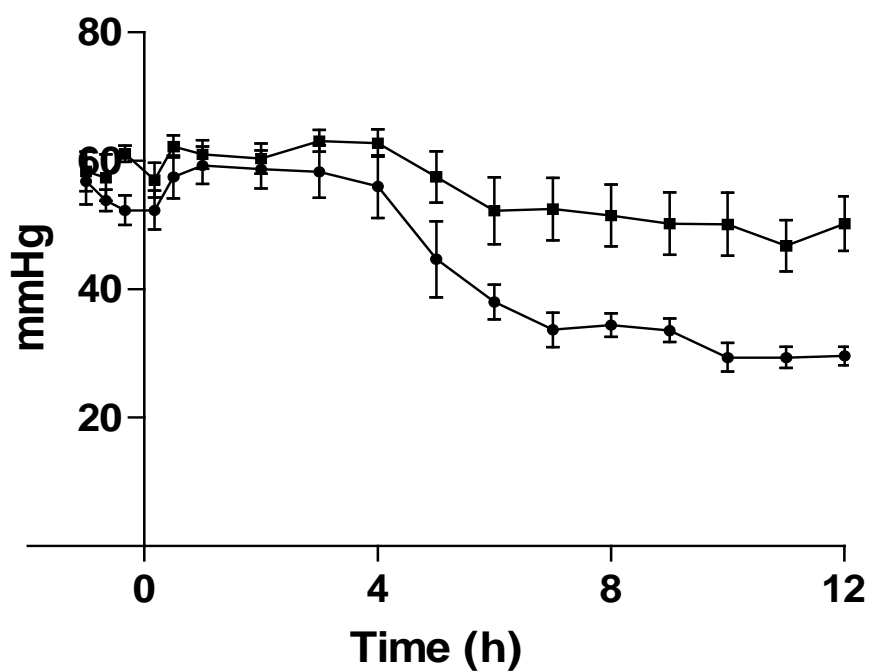
Blood and Cell Parameters.

– BE(B)	– Base excess
– Ca^{2+}	– Calcium concentration
– $\text{Ca}^{2+}(7.4)$	– Calcium concentration corrected for pH
– COHb	– Carboxyhaemoglobin
– HCO_3^-	– Bicarbonate concentration
– $\text{HCO}_3^- \text{ std}$	– Standard bicarbonate
– Hct	– Haematocrit
– Hgb	– Haemoglobin
– HHb	– Haemolysed haemoglobin
– IL-1 β	– Interleukin-1 β
– IL-6	– Interleukin-6
– IL-8	– Interleukin-8
– MetHb	– Methaemoglobin
– mcv	– Mean cell volume
– mch	– Mean cell haemoglobin
– mchc	– Mean cell haemoglobin concentration
– Na^+	– Sodium concentration
– O_2ct	– Oxygen content
– O_2Hb	– Oxyhaemoglobin
– pCO_2	– Partial pressure of carbon dioxide in blood
– $\text{pCO}_2 \text{ (t)}$	– Partial pressure of carbon dioxide in blood corrected for temperature
– pH	– Measure of acidity or basicity of a solution
– pH(t)	– Measure of acidity or basicity of a solution corrected for temperature
– PLT	– Platelets
– pO_2	– Partial pressure of oxygen in blood
– $\text{pO}_2 \text{ (t)}$	– Partial pressure of oxygen in blood corrected for temperature
– RBC	– Red blood cells
– RHb	– Reduced haemoglobin
– SO_2	– Oxygen saturation
– TCO_2	– Total carbon dioxide
– THb	– Total haemoglobin
– TNF- α	– Tumour necrosis factor alpha
– WBC	– White Blood Cells
– WBC(diffs)	– White blood cell differentials (identification of individual cells e.g. neutrophils, eosinophils, lymphocytes, Alveolar macrophages etc)

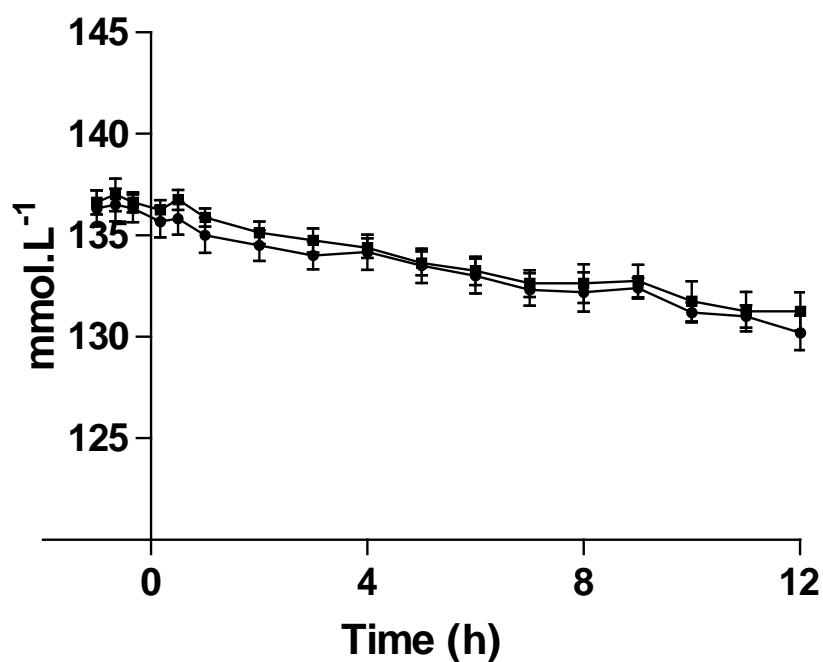
Blood Gas Parameters – Arterial

pCO₂

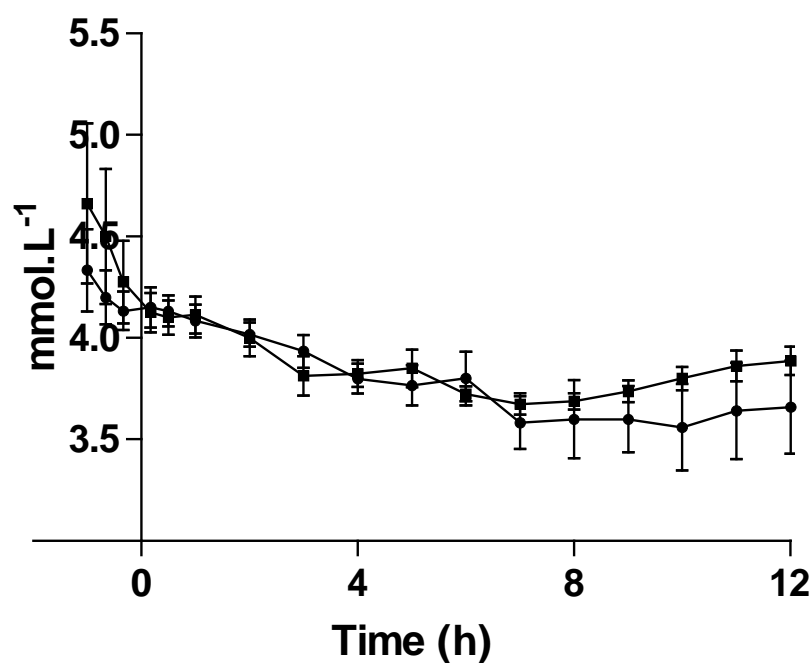
Changes in arterial carbon dioxide concentrations following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean ± SEM.

pO₂

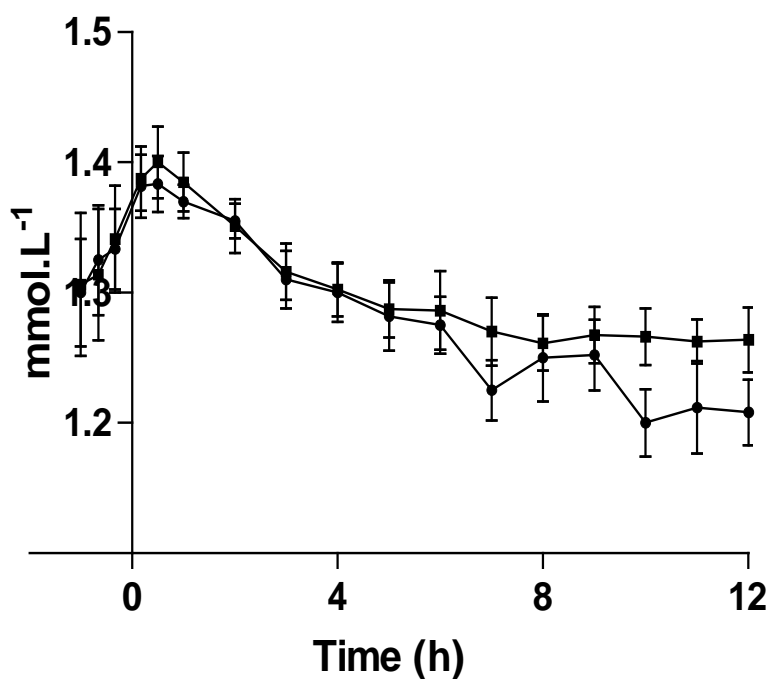
Changes in arterial oxygen concentrations following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean ± SEM.

Na⁺

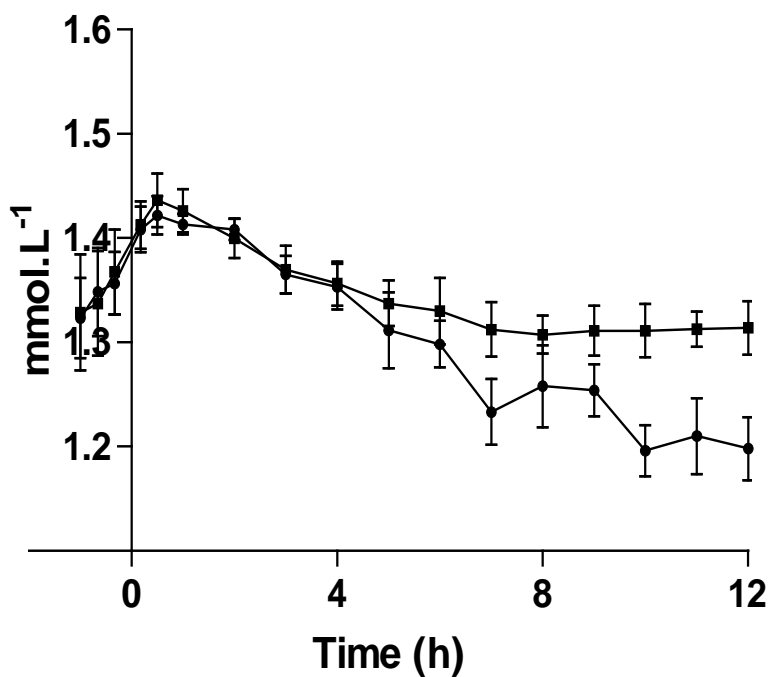
Changes in arterial sodium concentrations following exposure to HD: HD controls (●) and HD + NAC (■). Data are expressed as mean \pm SEM.

K⁺

Changes in arterial potassium concentrations following exposure to HD: HD controls (●) and HD + NAC (■). Data are expressed as mean \pm SEM.

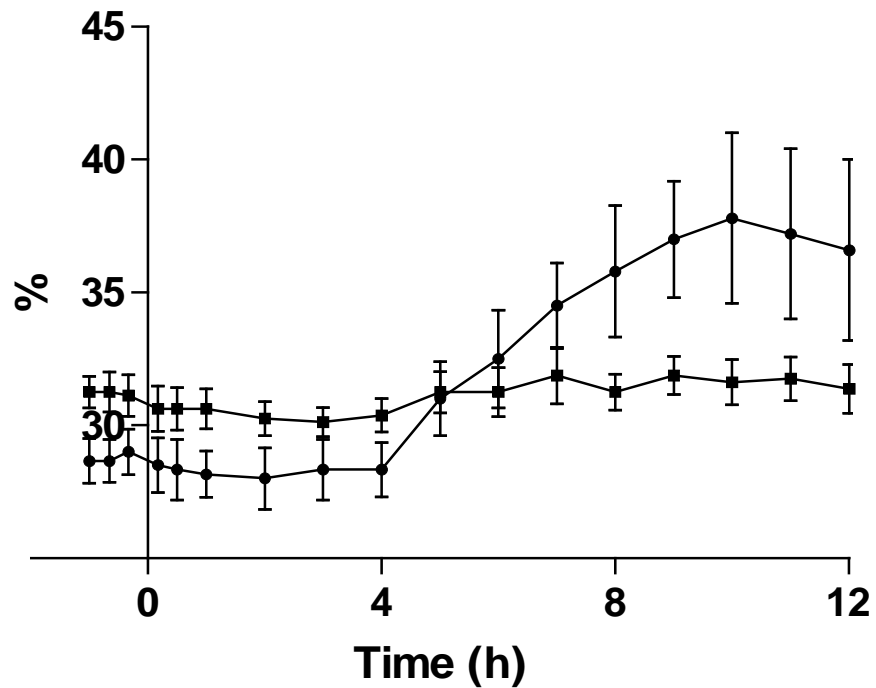
Ca⁺⁺

Changes in arterial calcium concentrations following exposure to HD: HD controls (●) and HD + NAC (■). Data are expressed as mean \pm SEM.

Ca⁺⁺(7.4)

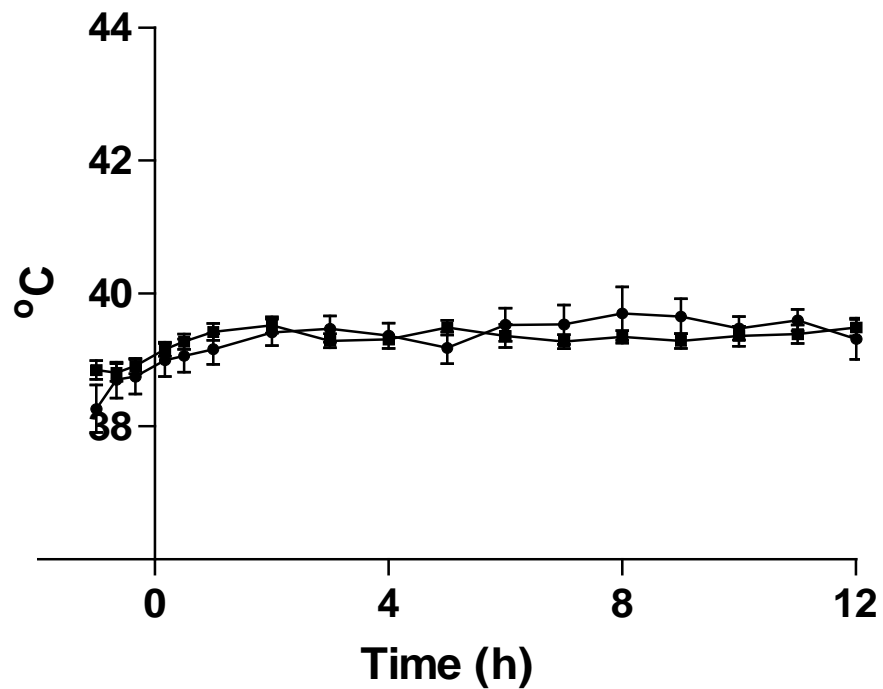
Changes in arterial calcium concentrations (corrected for pH) following exposure to HD: HD controls (●) and HD + NAC (■). Data are expressed as mean \pm SEM.

HCT



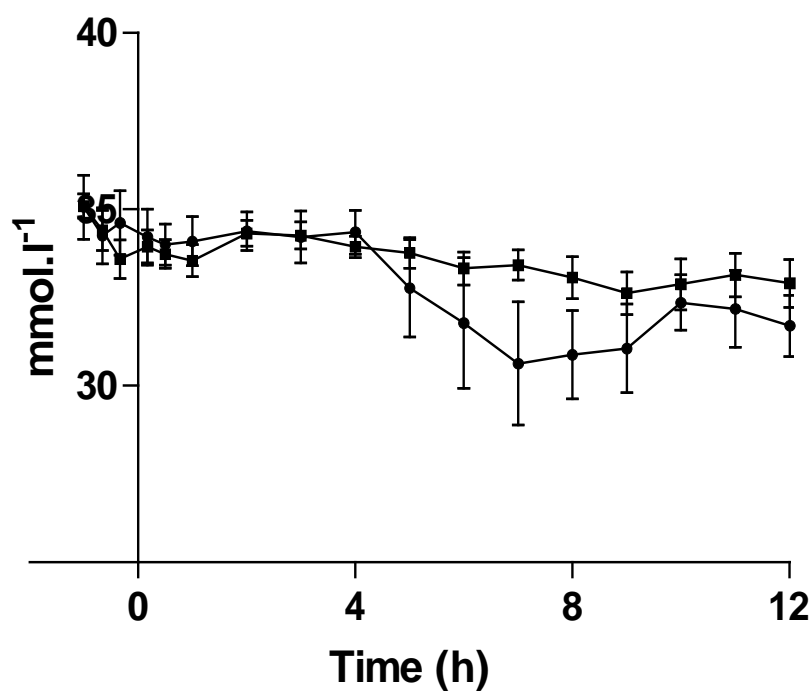
Changes in arterial haematocrit values following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

Temperature



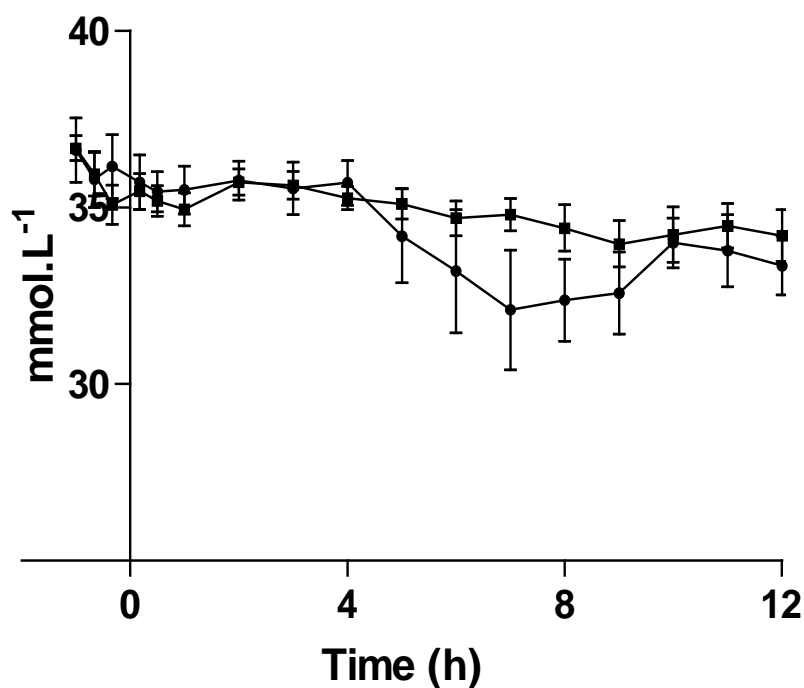
Changes in core temperature following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

HCO_3^-



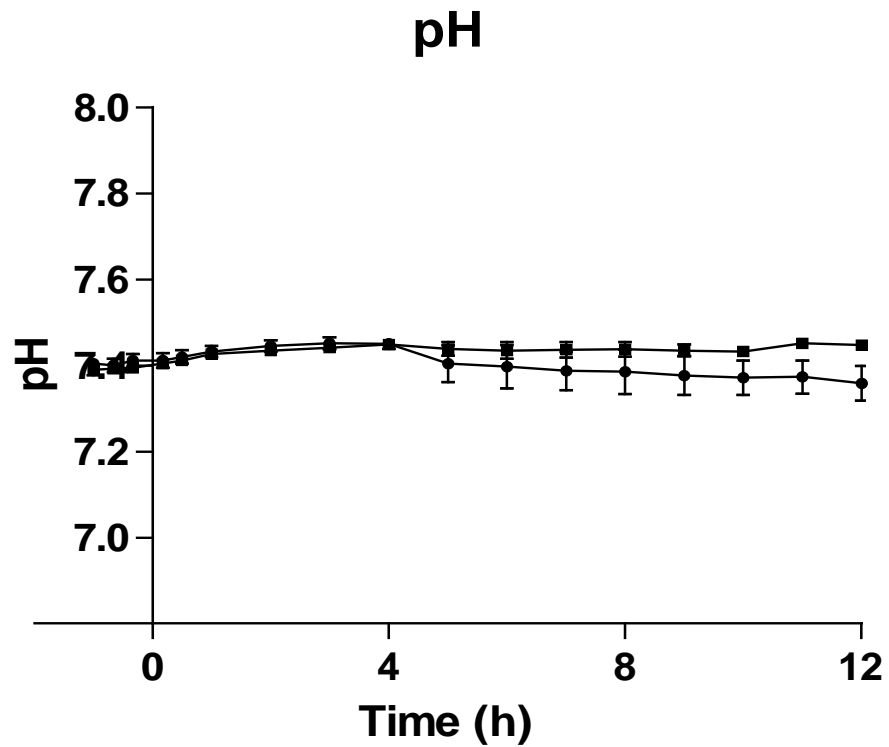
Changes in arterial bicarbonate concentrations following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

TCO_2

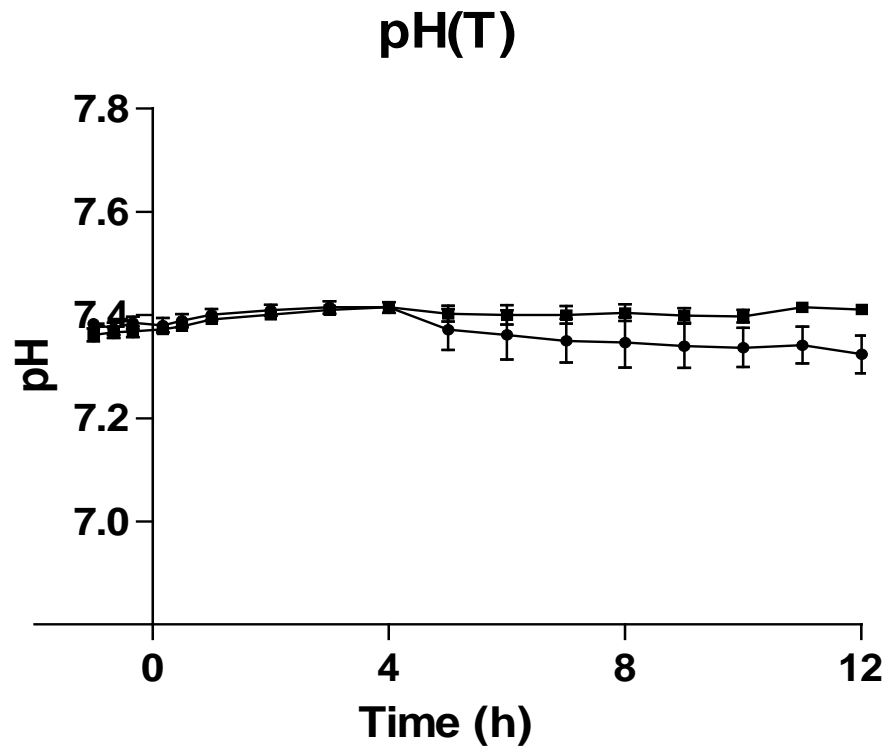


Changes in arterial total carbon dioxide concentrations following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

Blood Gas Parameters – Venous

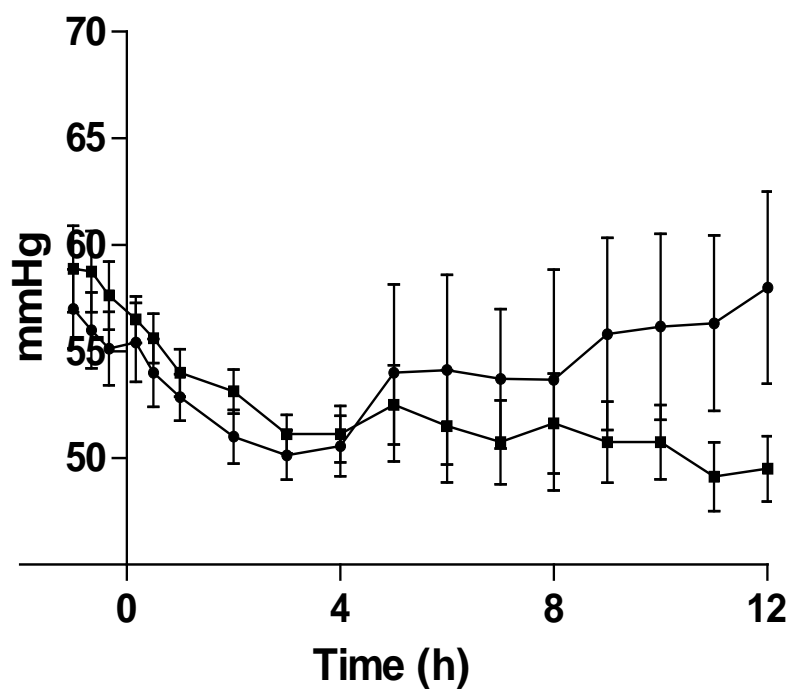


Changes in venous pH following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.



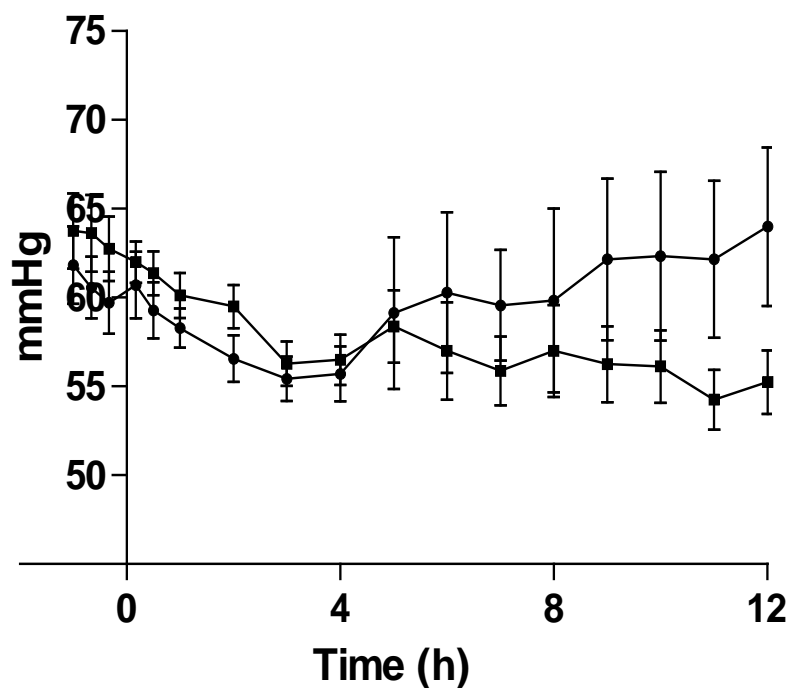
Changes in venous pH (corrected for temperature) following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

pCO₂

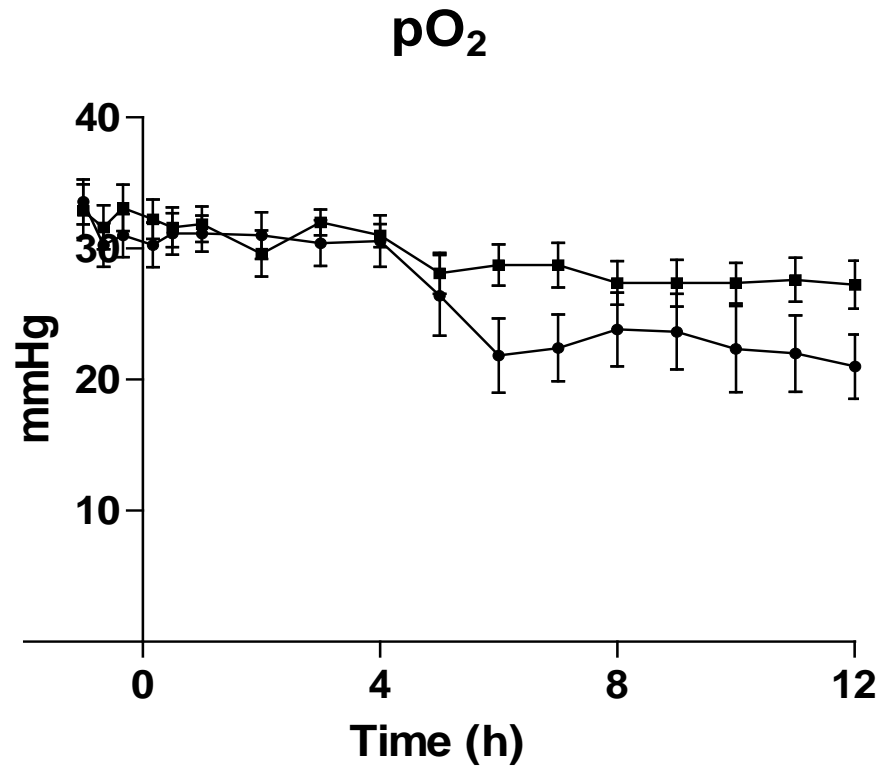


Changes in venous carbon dioxide concentrations following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

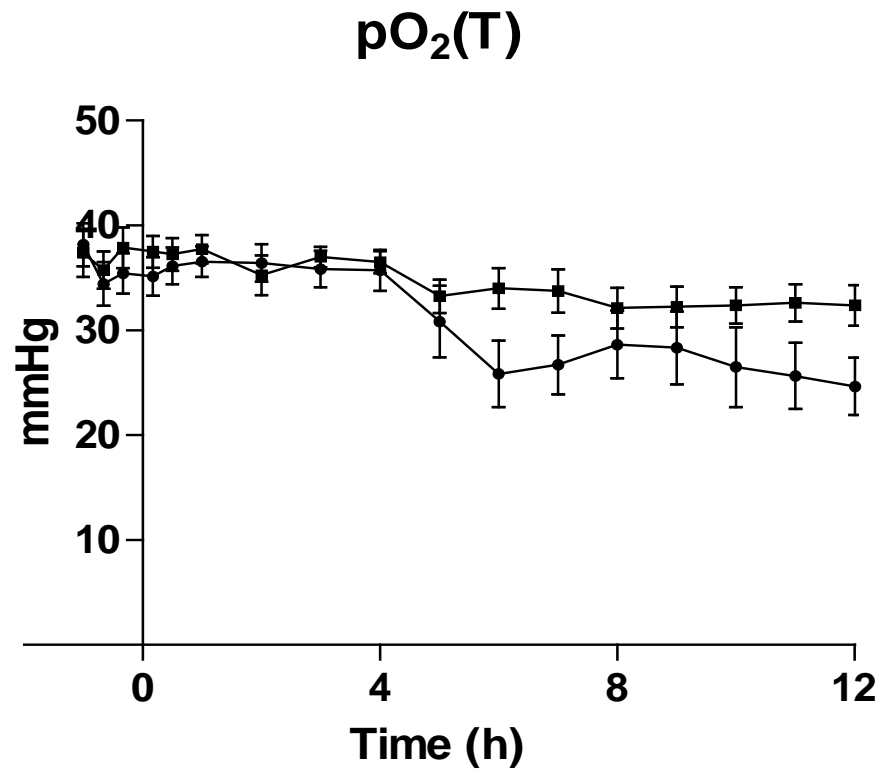
pCO₂(T)



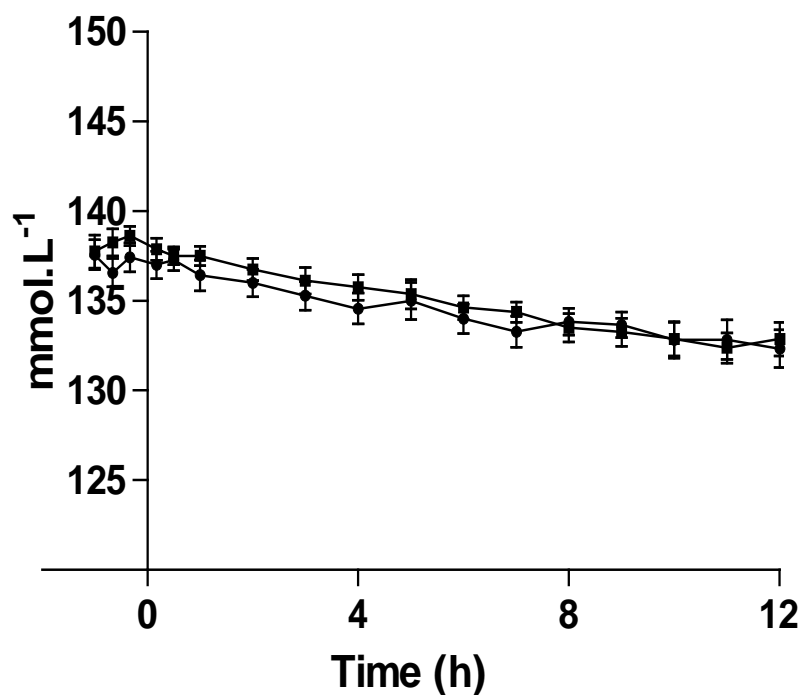
Changes in venous carbon dioxide concentrations (corrected for temperature) following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.



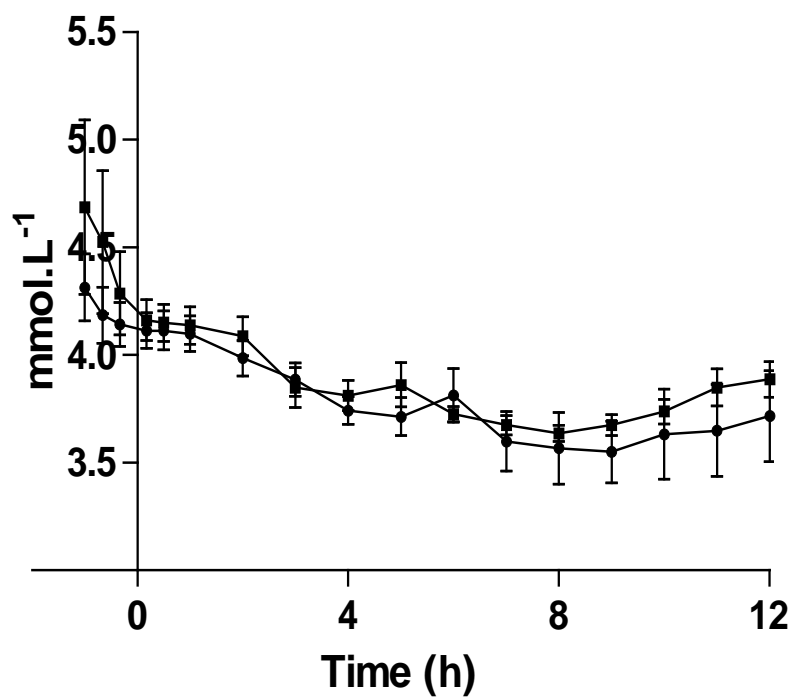
Changes in venous oxygen concentrations following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.



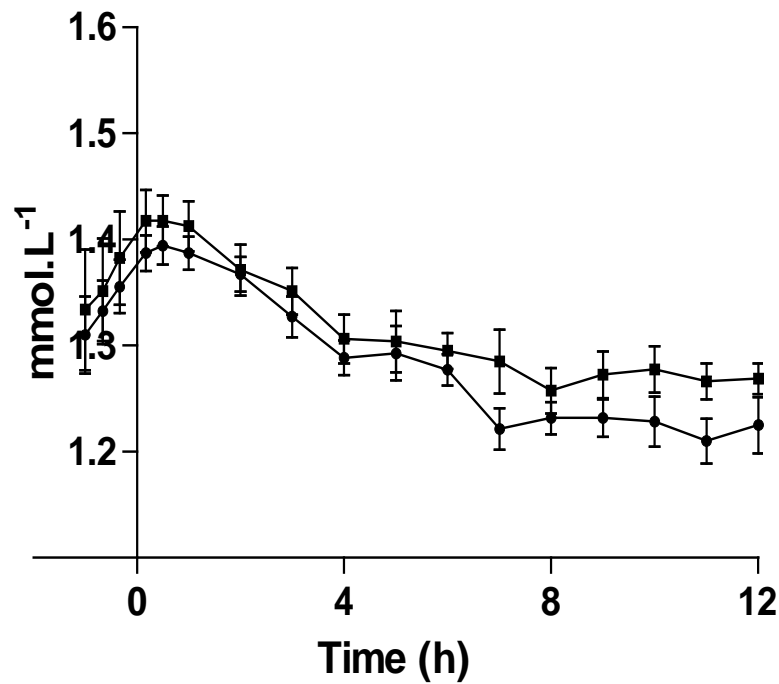
Changes in venous oxygen concentrations (corrected for temperature) following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

Na⁺

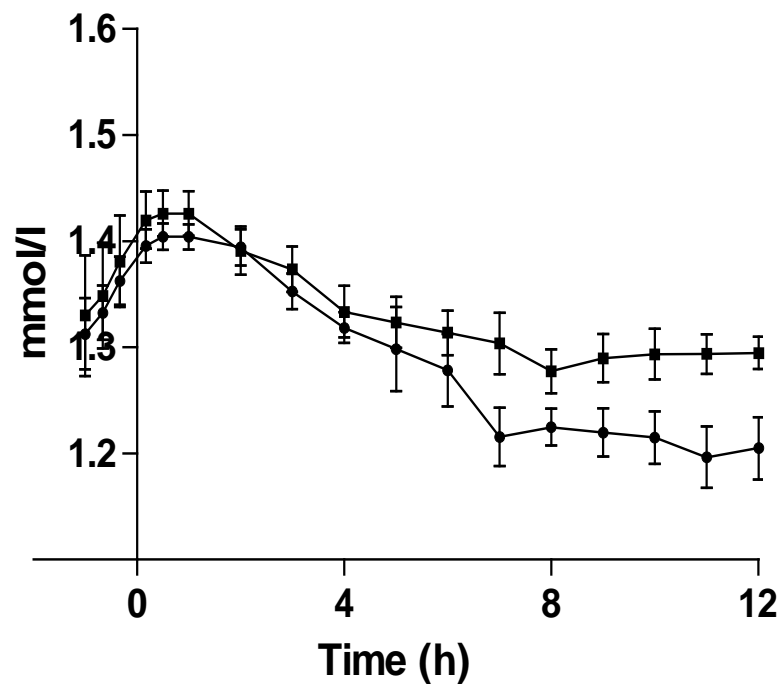
Changes in venous sodium concentrations following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

K⁺

Changes in venous potassium concentrations following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

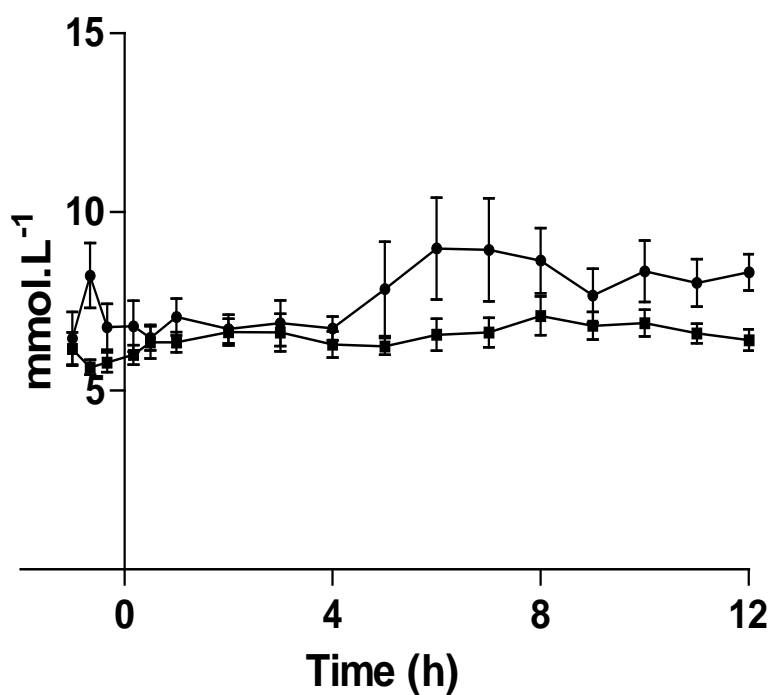
Ca⁺⁺

Changes in venous calcium concentrations following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

Ca⁺⁺(7.4)

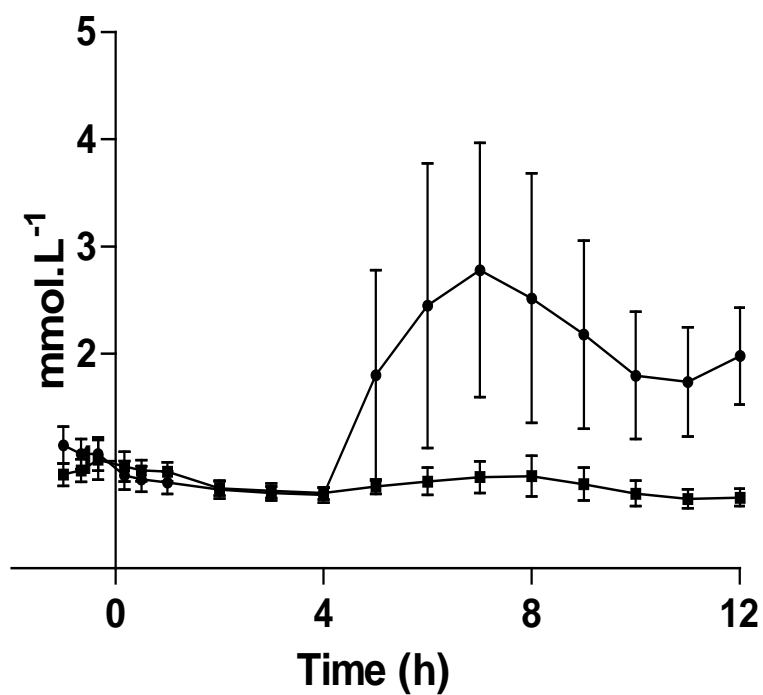
Changes in venous calcium concentrations (corrected for pH) following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

Glucose



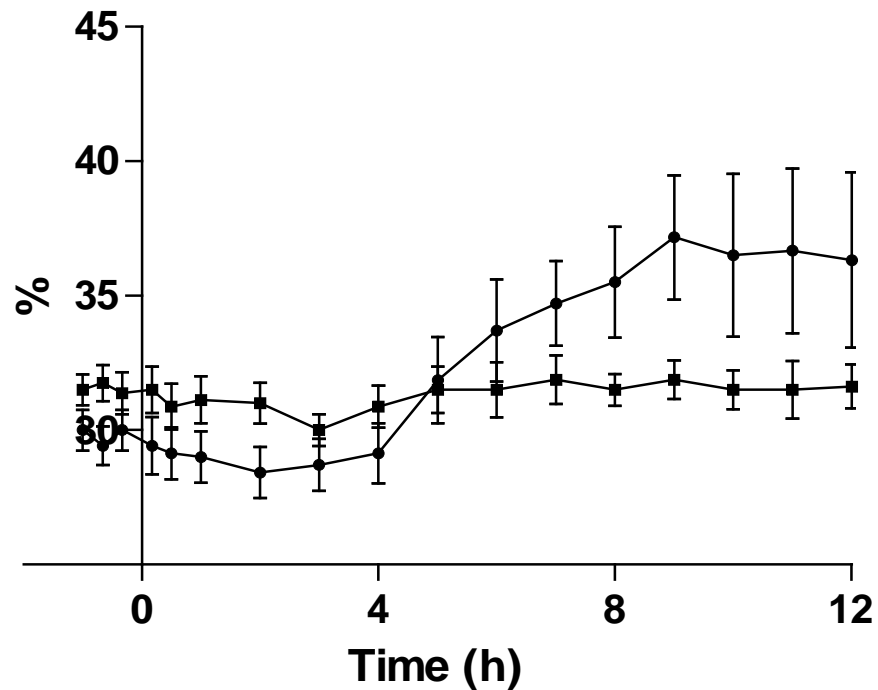
Changes in venous glucose concentrations following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

Lactate



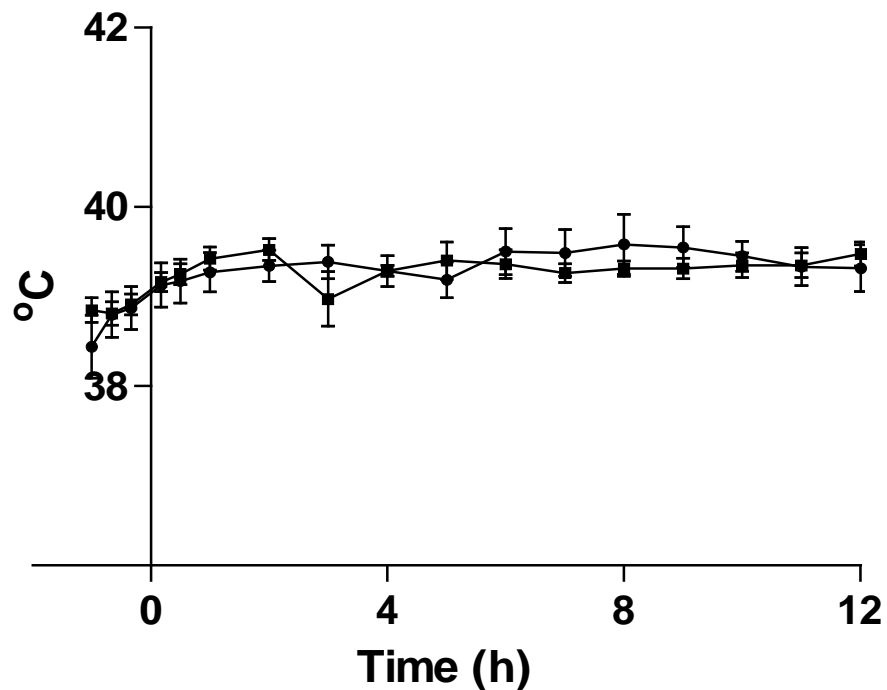
Changes in venous lactate concentrations following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

HCT



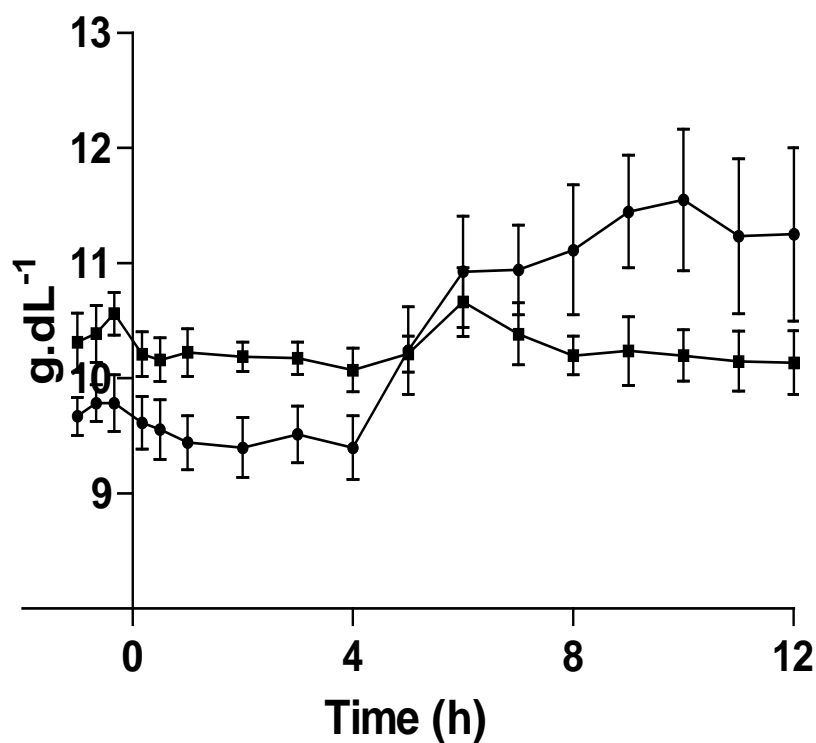
Changes in venous haematocrit values following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

Temp.



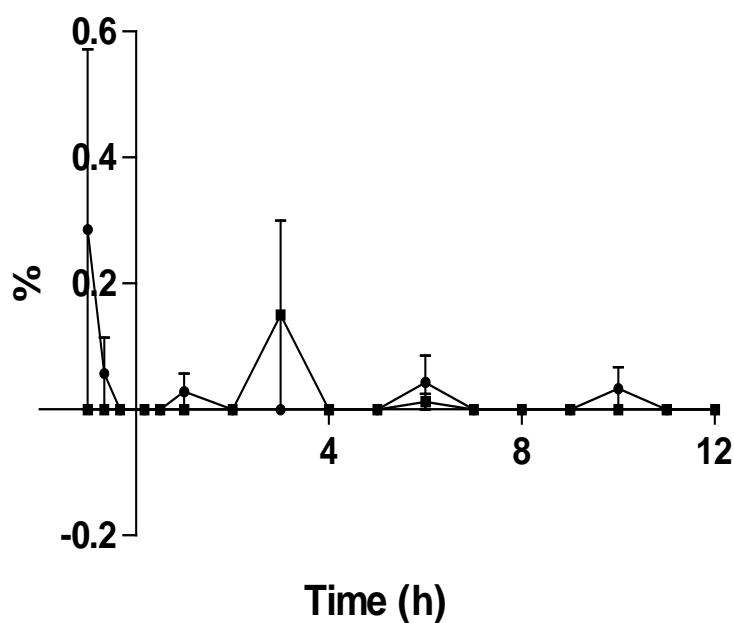
Changes in core temperature following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

THb

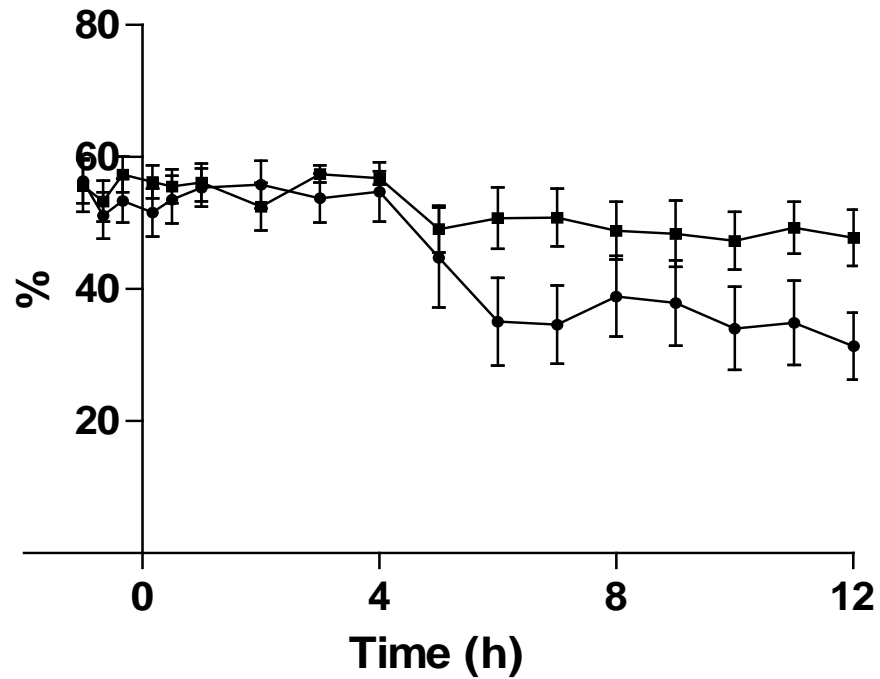


Changes in venous total haemoglobin following exposure to HD: HD controls (•) and HD + NAC (■). Data are expressed as mean \pm SEM.

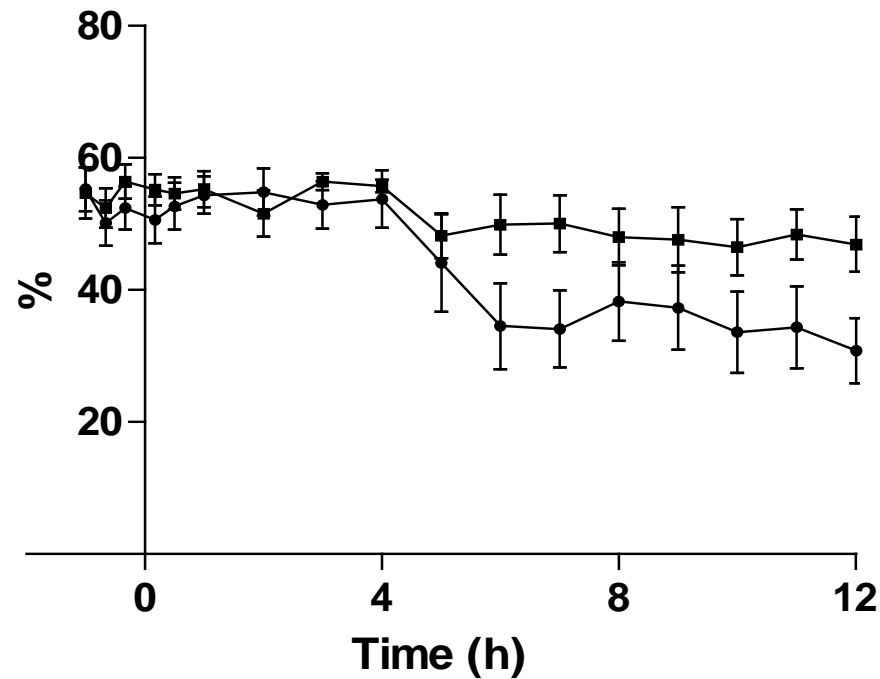
COHb



Changes in venous carboxyhaemoglobin following exposure to HD: HD controls (•) and HD + NAC (■). Data are expressed as mean \pm SEM.

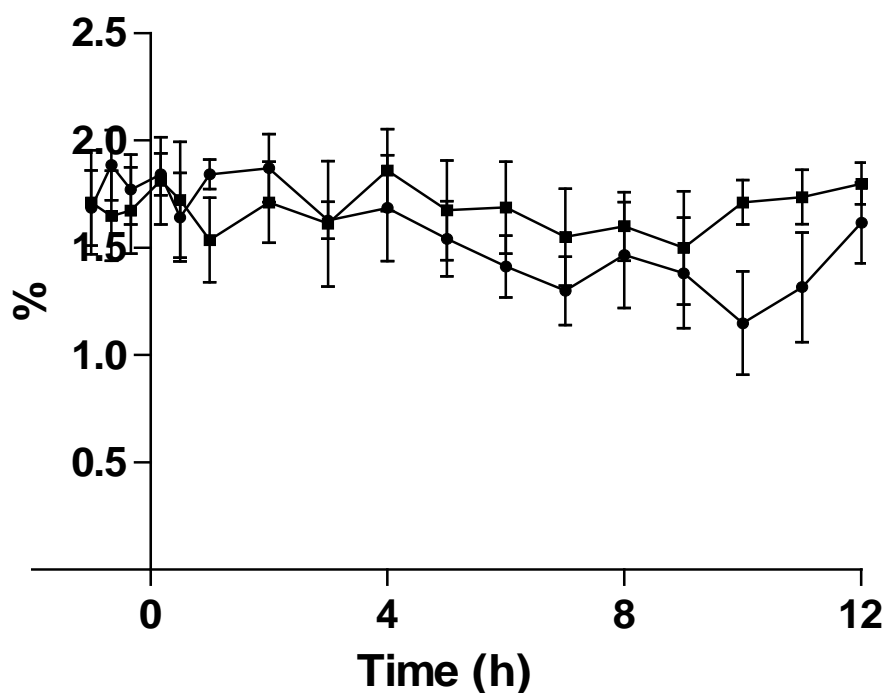
SO₂

Changes in venous oxygen saturations following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

O₂Hb

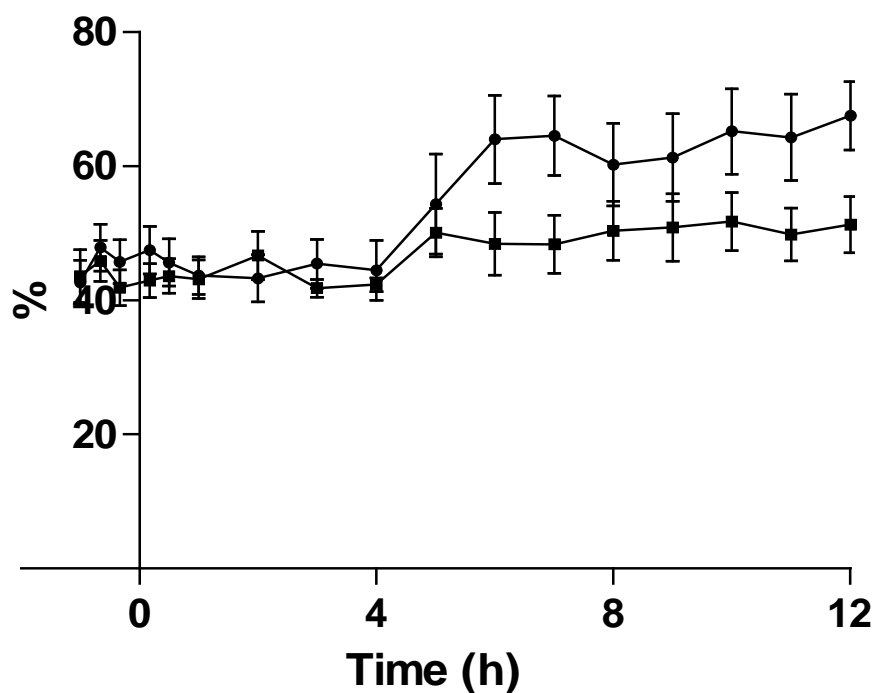
Changes in venous oxyhaemoglobin following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

MetHb

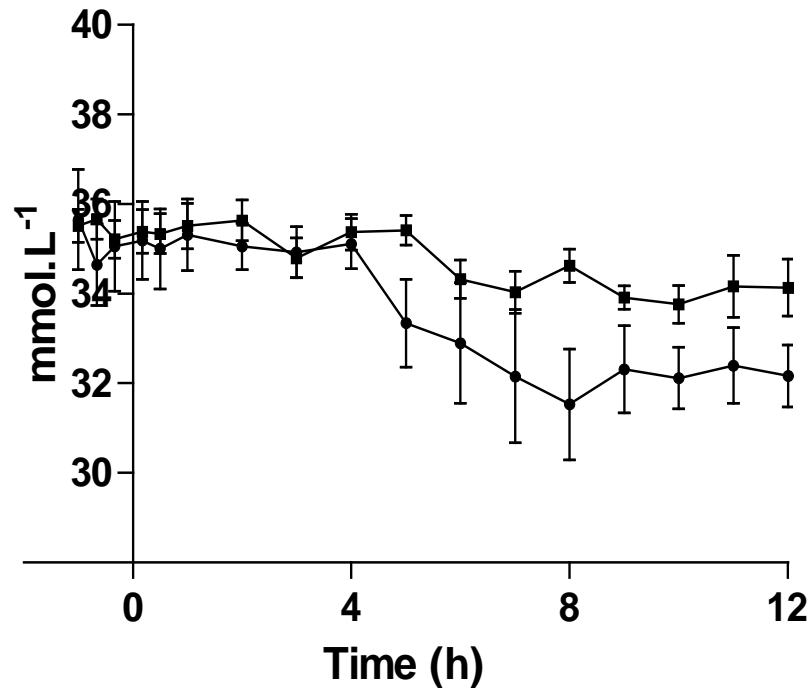


Changes in venous methaemoglobin following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

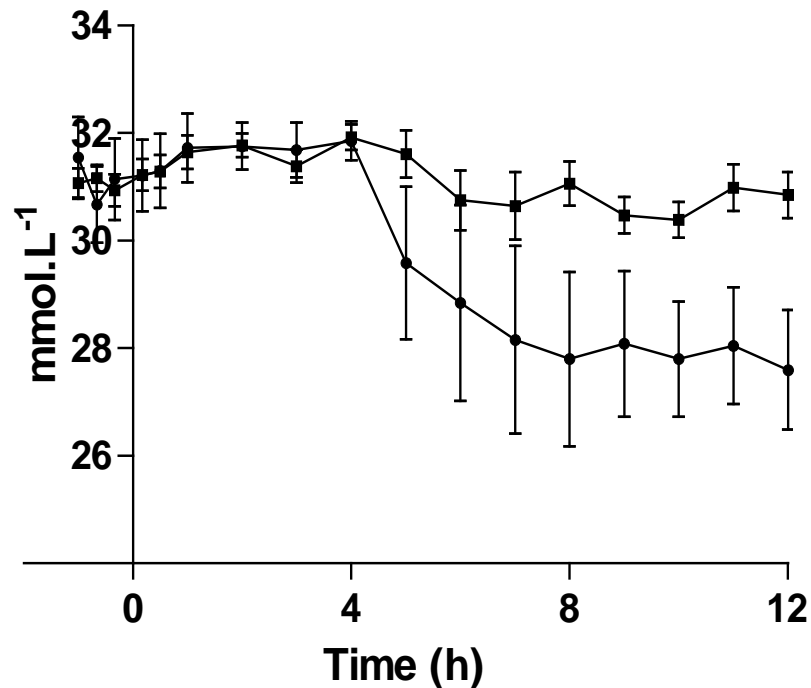
HHb



Changes in venous reduced haemoglobin following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

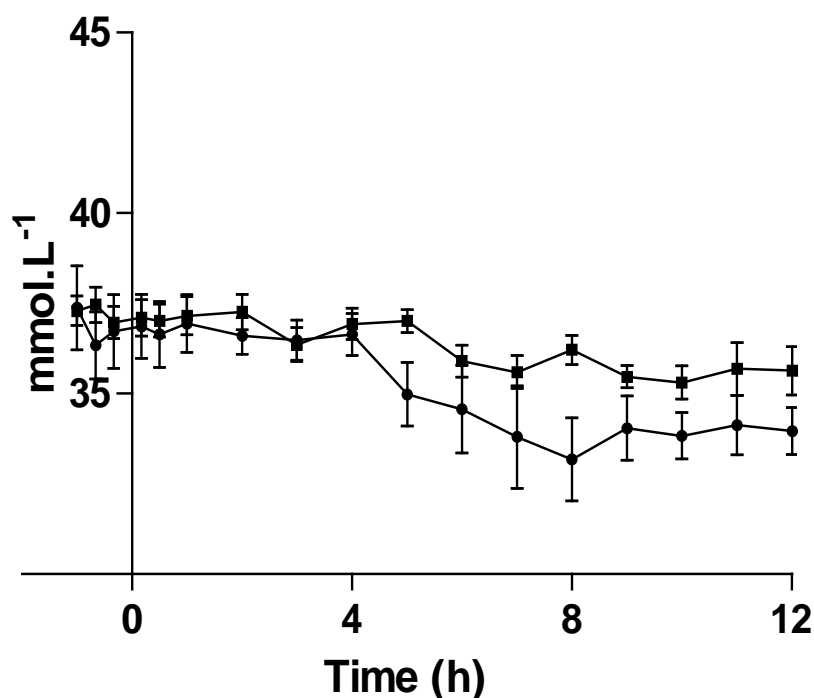
HCO₃⁻

Changes in venous bicarbonate concentrations following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean ± SEM.

HCO₃^{std}

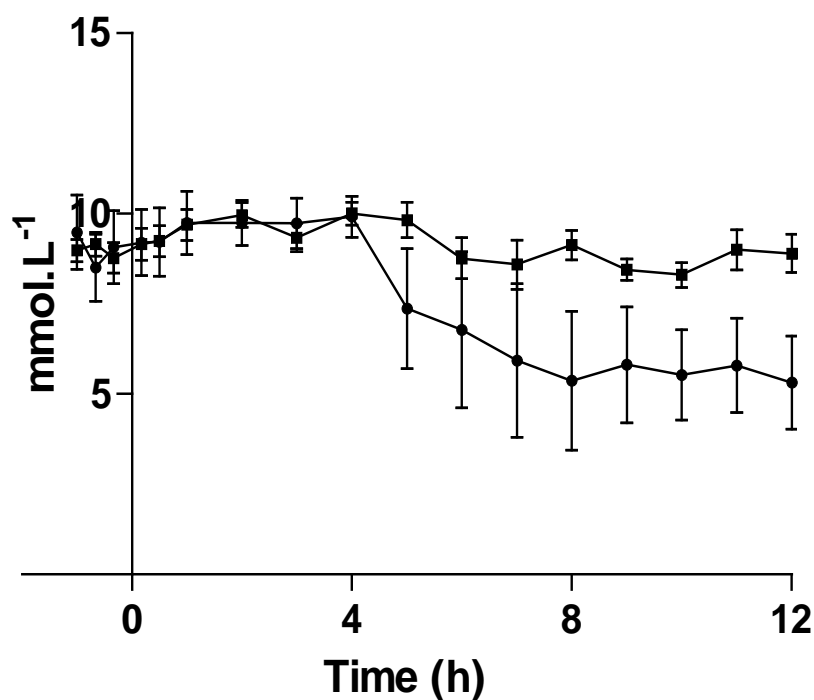
Changes in venous standard bicarbonate following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean ± SEM.

TCO₂



Changes in venous total carbon dioxide concentrations following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

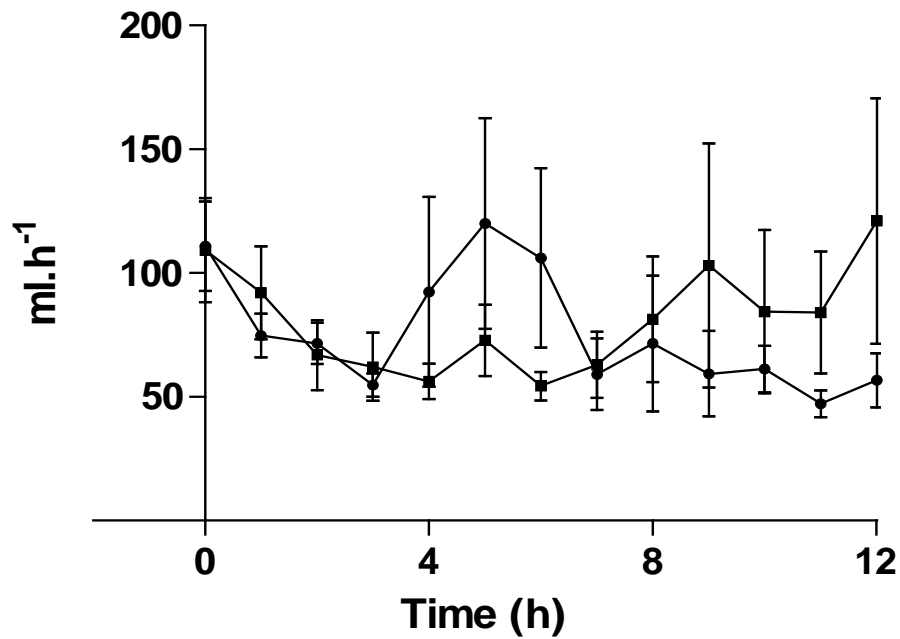
BE(B)



Changes in venous base excess following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

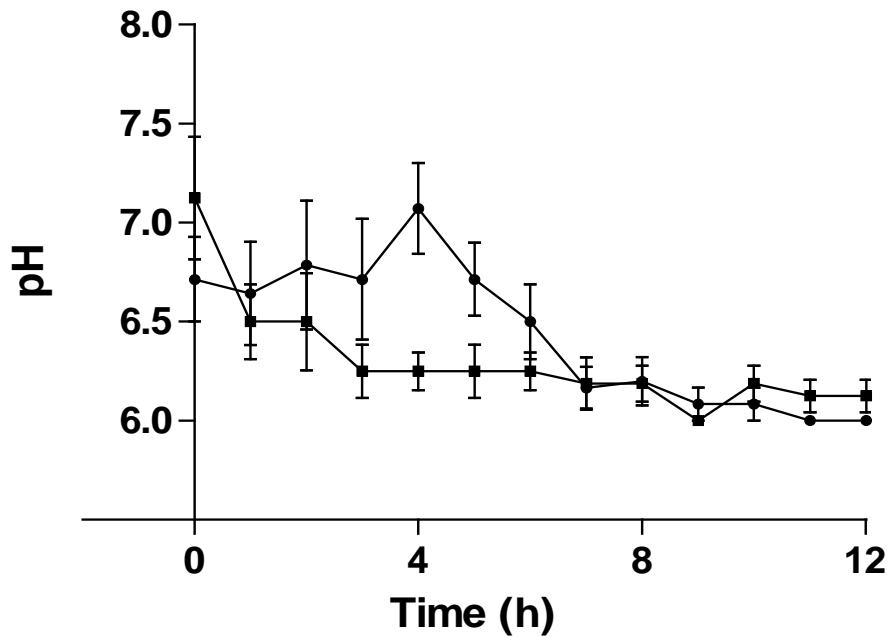
Urine

Volume



Changes in the volume of urine produced following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

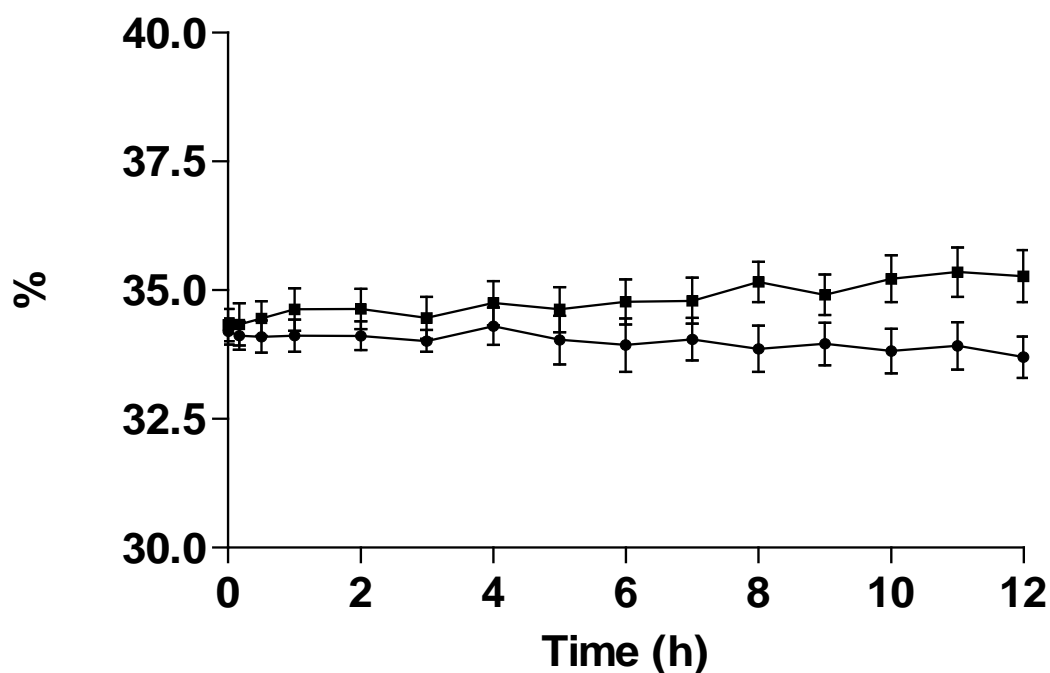
pH



Changes in the pH of urine following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

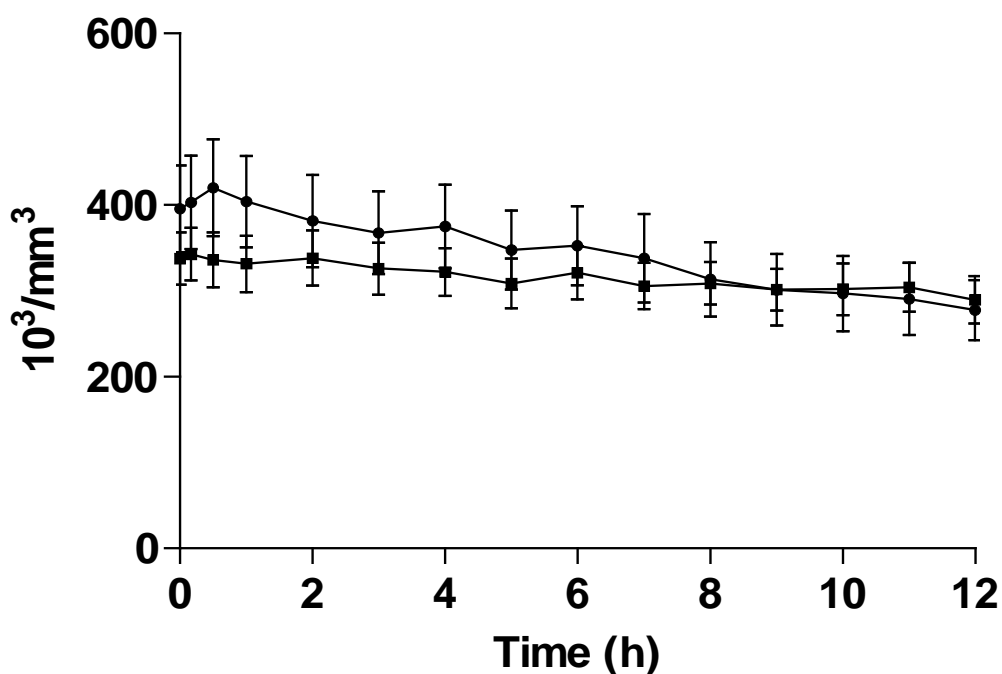
Haematology

MCHC

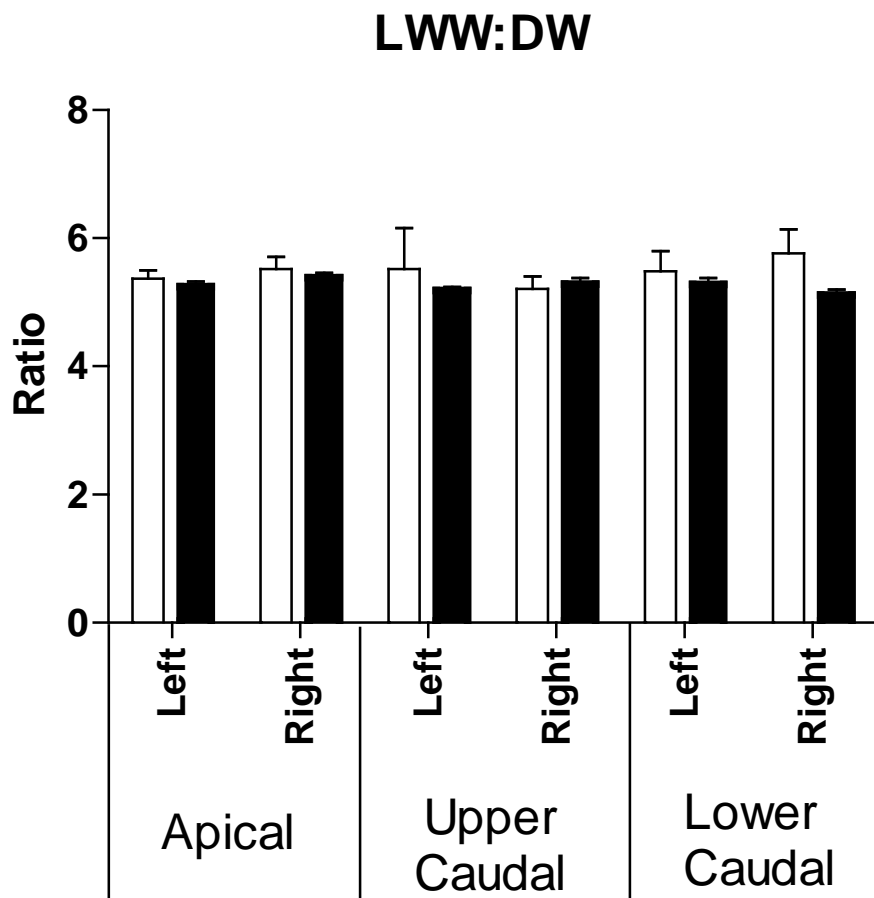


Changes in MCHC following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.

PLT

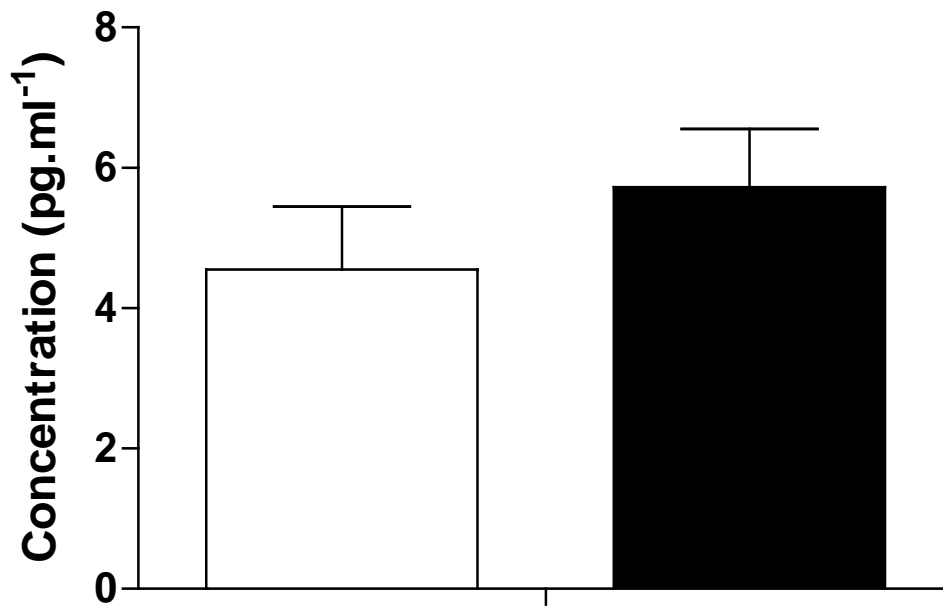


Changes in PLT following exposure to HD: HD controls (•) and HD + NAC (▪). Data are expressed as mean \pm SEM.



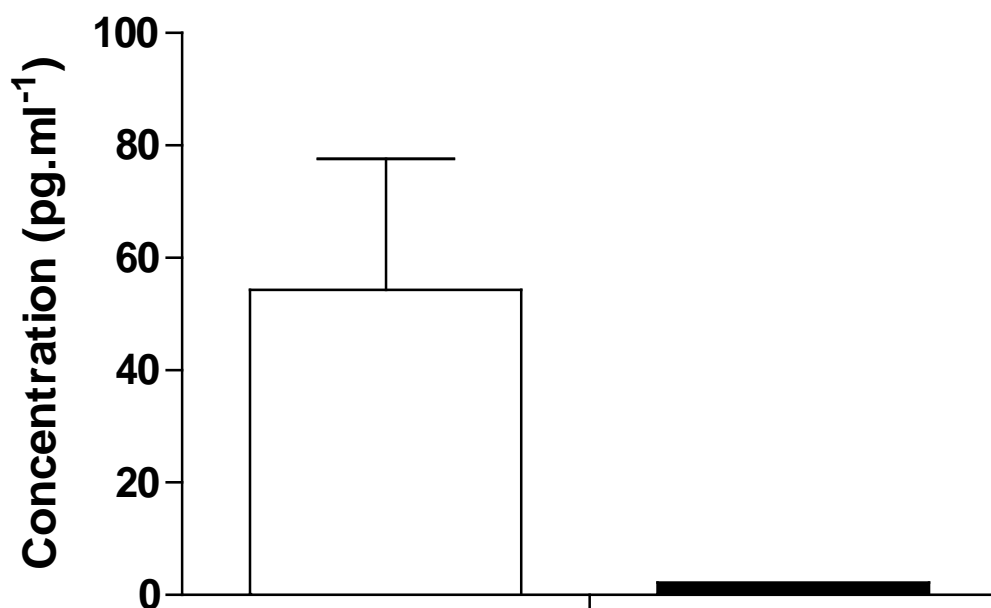
Changes in lung wet weight to dry weight ratio following exposure to HD: HD controls (□) and HD + NAC (■). Data are expressed as mean \pm SEM.

Inflammatory Mediators

IL-1 β (Lavage)

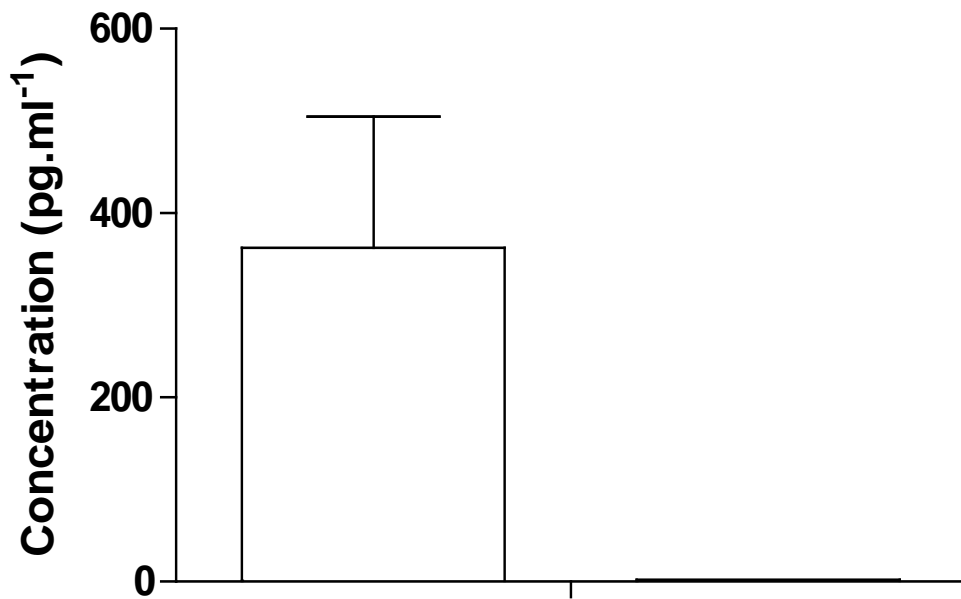
Changes in IL-1 β (lavage) following exposure to HD: HD controls (□) and HD + NAC (■). Data are expressed as mean \pm SEM.

IL-6 (Lavage)



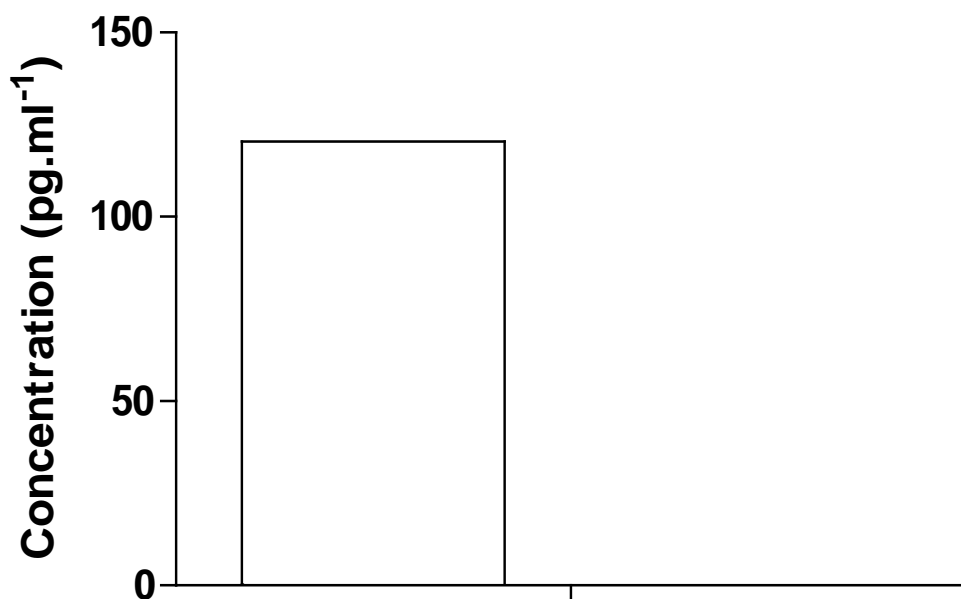
Changes in IL-6 (lavage) following exposure to HD: HD controls (□) and HD + NAC (■). Data are expressed as mean \pm SEM.

IL-8 (Lavage)



Changes in IL-8 (lavage) following exposure to HD: HD controls (□) and HD + NAC (■). Data are expressed as mean \pm SEM.

TNF- α (Lavage)



Changes in TNF- α (lavage) following exposure to HD: HD controls (□) and HD + NAC (■). Data are expressed as mean \pm SEM.

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External distribution

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| 3. | Programme Lead CBR Protect | Dr Phil Packer | Electronic |
| 4. | Project File | | Electronic |
| 5. | Author | Mrs B Jugg | Electronic |

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Report documentation page

v1.0

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4b.	Report national caveats: UK UNCLASSIFIED		
4c.	Report descriptor:		
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17a. Abstract:	<p>Sulphur mustard (HD) is best known as a blister agent but the majority of deaths are associated with pulmonary damage. Inhalation of HD vapour can cause life threatening lung injury for which there is currently no specific medical therapy, available treatment remaining largely supportive. This treatment often requires intensive care facilities which may become overwhelmed in mass casualty events, and may be of limited benefit in severe cases. There therefore remains a need for evidence based treatment strategies to inform both military and civilian medical response teams on the most appropriate treatment for chemically induced lung injury. A clinically relevant in vivo large animal model has been established. Exposure to 100 µg.kg⁻¹ inhaled HD resulted in changes in oxygenation and acid/base balance in HD exposed animals as well as pathology consistent with anecdotal human poisoning.</p> <p>The final phase of the programme investigated an appropriate treatment strategy against inhaled HD-induced lung injury in the established model. The thiol compound, N-acetyl-L-cysteine (NAC - Mucomyst™) was chosen due to its anti-oxidant and mucolytic effects, administered via the inhaled route. NAC had been shown to present potential therapeutic benefit against HD-induced lung injury in a small animal model (Anderson D <i>et al</i>; 2000). In our model results indicate physiological benefit following inhaled NAC therapy; further studies are warranted to look at NAC in combination with other beneficial therapies.</p>	
17b. Abstract UK protective marking:	UK UNCLASSIFIED	
17c. Abstract national caveats:	UK UNCLASSIFIED	
17d. Abstract descriptor:		
18. Keywords:	Acute lung injury (ALI), inflammation, inhalation, mono-sulfoxide, physiology, thiol	
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19f.	<input type="checkbox"/>	<input type="checkbox"/> UK MOD has no rights of distribution
19b.	<input type="checkbox"/>	<input checked="" type="checkbox"/> Can be distributed to UK MOD and its agencies
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19g. Additional announcement:		
19h. Additional availability:		
19i. Release Authority role:	Dr Paul Rice, Chief Scientist, Biomedical Sciences Dept., DSTL.	

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The Toxicity of Inhaled Sulfur Mustard in a Large Animal Model

Smith AJ, Fairhall SJ, Mann T, Sciuto A and Jugg BJA

Dstl/TR50669
July 2010e

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Executive summary

Exposure of military and civilian populations to inhaled toxic chemicals can take place as a result of deliberate release (warfare, terrorism) or following accidental releases from industrial concerns or transported chemicals. Sulfur mustard (HD) is best known as a blister agent but the majority of deaths are associated with pulmonary damage. Inhalation of HD vapor can cause life threatening lung injury for which there is currently no specific medical therapy, available treatment remaining largely supportive. This treatment often requires intensive care facilities which may become overwhelmed in mass casualty events, and may be of limited benefit in severe cases (Marrs 1996). There therefore remains a need for evidence based treatment strategies to inform both military and civilian medical response teams on the most appropriate treatment for chemically induced lung injury. A reproducible, characterized *in vivo* model is required to produce this evidence base for novel therapies targeting HD-induced lung injury.

Previous research has demonstrated a limitation in the use of small animals to model the effects of inhaled chemicals in man. Small animals exposed to inhaled HD showed severe necrosis of the upper respiratory tract and corneal epithelium. Histopathological analysis revealed pulmonary damage was much less severe than expected, and inconsistent with available evidence on human poisonings in World War I (WW1). Most of the HD had acted in the nasopharynx which has a very large surface area compared to that of humans, with very little reaching the deep regions of the lung. Small animals are obligate nasal breathers, while humans breathe through both the nose and mouth. During periods of exercise, or when under stress, humans breathe more through the mouth which is likely to increase the HD delivery to the deep lung. Therefore, an animal model in which the upper respiratory tract is bypassed will more accurately reflect human exposure under stress, such as in combat or when running to escape a contaminated area.

The aim of this programme was to establish a clinically relevant, reproducible large animal model of HD exposure. Intubation of these animals and exposure directly via the endotracheal (ET) tube better represents human exposure via the nose and mouth compared with small animal models. Initially, a dose ranging study was established to identify the doses of HD that produced a range of lung injuries, out to 6 hours post exposure. This was followed by a 12 hour toxicology study that investigated the physiological and pathological effects of a moderate, but still potentially lethal, dose of HD. This model allowed an assessment of the toxicology of HD poisoning with clinically relevant physiological parameters in an intensive care setting. This report covers in a single publication all of the data recorded from both the 6 hour dose ranging study and the 12 hour toxicology study, which compliments the peer reviewed publications (Fairhall *et al.*, 2008, Fairhall *et al.*, 2010).

6 hour study

Anesthetized, spontaneously breathing large white pigs were exposed to inhaled doses of HD vapor of 60 $\mu\text{g.kg}^{-1}$ (n = 5, Low), 100 $\mu\text{g.kg}^{-1}$ (n = 5, Medium) or 150 $\mu\text{g.kg}^{-1}$ (n = 5, High), or to air (n = 4), and monitored for 6 hours. Cardiovascular and respiratory parameters were recorded continuously throughout the experiment. Blood and

bronchoalveolar lavage fluid (BALF) were collected to allow blood gas and hematology analysis, and to assay for lung inflammatory cells and mediators. Urine was collected and analyzed for HD metabolites. Tissue samples were taken *post mortem* and processed for histopathological analysis.

Normal lung physiology was maintained in air control animals (lying supine and spontaneously breathing) for 6 hours. There was a statistically significant increase in shunt fraction across all three HD exposed groups when compared to air controls at 3 - 6 hours post exposure. HD exposed animals were increasingly hypoxemic with respiratory acidosis. The mono-sulfoxide β -lyase metabolite of HD (1-methylsulfinyl-2-[2(methylthio)ethylsulfonyl]ethane], MSMTESE), was detected in urine from 2 hours post exposure. Pathological examination revealed necrosis and erosion of the tracheal epithelium in medium and high HD exposed groups.

12 hour study

Anesthetized, spontaneously breathing large white pigs were exposed to HD vapor at 60 $\mu\text{g.kg}^{-1}$ (n = 3, Low), 100 $\mu\text{g.kg}^{-1}$ (n = 7, Medium), or to air (n = 6), and monitored for 12 hours. Cardiovascular and respiratory parameters were recorded continuously throughout the experiment. Blood and BALF were collected to allow blood gas and hematology analysis, and to assay for lung inflammatory cells and mediators. Urine was collected and analyzed for HD metabolites. Tissue samples were taken *post mortem* and processed for histopathological analysis.

Normal lung physiology was maintained in air controls and low dose HD exposed animals (lying supine and spontaneously breathing) for 12 hours. In the medium dose group there were statistically significant changes in oxygenation parameters. Arterial blood oxygenation (PaO_2) decreased from 6 - 12 hours, with an associated decrease in oxyhemoglobin from 6 - 12 hours. These animals showed increased respiratory acidosis with significant decreases in arterial base excess, arterial bicarbonate and sodium concentrations as well as increased hematocrit. MSMTESE was detected in urine 2 hours post exposure with levels increasing until the end of the 12 hour study. Pathological examination revealed areas of edema, inflammatory cell infiltration and necrosis of the tracheal epithelium in the medium HD exposed group. These findings are consistent with those seen in the early stages of acute lung injury (ALI).

Plasma, BALF and urine samples from the 12 hour study were transferred to the University of Portsmouth School of Pharmacy and Biomedical Sciences, for analysis of desmosine, collagenase activity, elastase activity and myeloperoxidase activity. The results provide evidence of sulfur mustard-induced neutrophil activation in the lung and elastase-mediated breakdown of the matrix protein elastin, which can be monitored as desmosine in urine. This provides evidence for the presence of activated neutrophils in the lung. Breakdown of lung matrix by Matrix Metalloproteinases may contribute to sulfur mustard-induced lung injury. This may provide a potential therapeutic option for intervention.

A reproducible model of lung injury following HD exposure has been established. Assessment of the toxicological profile of HD exposure has identified changes consistent with anecdotal evidence from human poisoning, allowing confidence to assess potential

treatment strategies. The early identification of urinary MSMTESE has the potential to be a diagnostic and prognostic indicator of HD exposure and treatment efficacy.

This model will now be used to evaluate the efficacy of potential intensive care treatment strategies and commercial off the shelf (COTS) drugs. This will provide an evidence based strategy to military and civilian medical personnel for the treatment of chemically induced lung injury.

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1 Introduction

The use of sulfur mustard (bis(2-chloroethyl)sulphide; HD) as a chemical warfare (CW) agent remains of concern to the Armed Forces. Since its first use in World War 1 (WW1; Prentiss, 1937) HD has been used in numerous locations across the world by aggressors (Stockholm International Peace Research Institute [SIPRI], 1971). This includes during the Iran-Iraq conflict in the 1980s where the use of HD resulted in over 100,000 medical casualties (Hefazi *et al.*, 2005; Pechura and Rall, 1993).

Though primarily a dermal vesicant, it is the inhaled effects of HD which account for the majority of deaths following HD poisoning. Indeed, the majority of deaths from HD poisoning during WW1 had some degree of pulmonary involvement (Prentiss, 1937). The respiratory effects following exposure to HD vapor range from mild effects such as an unproductive cough, bronchitis, necrosis of the respiratory epithelium, bronchopneumonia, pulmonary edema and respiratory failure; often a consequence of complications such as infection (Kehe and Szinicz, 2005; Russell *et al.*, 2006).

HD is a successful CW agent because of its low threshold of effect, causing incapacitation at a dose of approximately 0.0065 mg.l^{-1} following a 60 minute exposure (Prentiss, 1937). In addition, it also has a high odor detection threshold (0.6 mg.m^{-3}), making it difficult to ascertain whether one has been exposed (Pechura and Rall, 1993). Together, these properties, coupled with the persistency of HD and the delay in the onset of symptoms of approximately four hours, make the development of an effective therapy for HD poisoning of utmost importance. To date, no specific treatment for HD poisoning exists. A reliable and reproducible *in vivo* model of HD lung injury needs to be established in which potentially effective treatments could then be evaluated.

Although the effects of HD have been studied *in vitro*, the biochemical, physiological and toxicological acute effects of HD lung injury are not fully understood. A small number of studies have been carried out to investigate HD induced lung injury in whole animal models. These found that exposure to HD vapor resulted in the formation of pseudomembranes covering interior surfaces of the tracheobronchial tree. HD is readily absorbed within the respiratory tract and the estimated LCt_{50} in mice was reported to be $101 \text{ mg.min.m}^{-3}$ for a ten minute exposure (Winternitz and Finney, 1920; Cameron *et al.*, 1946; Box and Cullumbine, 1947). However, as rodents are obligate nasal breathers it is difficult to extrapolate the results to man, who is not. HD injury in rodents is more severe within the upper respiratory tract (URT) whereas in humans it is the lower respiratory tract (LRT) that is most affected. It is therefore essential to find a species more directly comparable to man so that any data derived can be extrapolated with greater confidence.

This laboratory has established a large white pig model of HD induced lung injury. The large white female pig was chosen for this study as it is a well studied and understood species. Its lung physiology is comparable to that of man (Cramer *et al.*, 1994; Getty., 1975) and its size means that conventional human medical equipment can be utilized due to similarities in the tracheobronchial and vascular architectures. The pig has been widely used as a model of acute lung injury (ALI) caused by inhaled agents such as

chlorine (Gunnarsson *et al.*, 1998) and phosgene (CG) (Brown *et al.*, 2002, 2006), by bacterial infection (Dehring *et al.*, 1983) and by physical trauma (Garner *et al.*, 2009;Thörne *et al.*, 1989). The use of the pig also allows the URT to be bypassed, enabling the effects of HD when delivered directly to the lung to be investigated. Our laboratory has proven expertise in using this animal model (Brown *et al.* 2002, 2006). A novel exposure system for the delivery of HD vapor directly to the lung has previously been reported (Fairhall *et al.*, 2008) and preliminary respiratory data presented.

The aim of the work described here was to expose the anaesthetized, spontaneously breathing large white pig to a range of doses of HD vapor directly to the lung in a reliable and quantitatively reproducible manner. The effects of exposure on lung physiology, hematology, inflammatory markers and pathology were investigated. The primary outcome measures for this study were mortality and arterial blood oxygenation, however, a number of secondary outcome measures were also evaluated, including cardiovascular and pathological indices. This study will facilitate the future development of therapeutic strategies against HD induced lung injury.

2 Method

2.1 Animals and surgical preparation

All animal work was carried out in accordance with the UK Animals (Scientific Procedures) Act 1986, and was approved by Ethical Review at Dstl, Porton Down, UK.

Large white female pigs (47 - 55 kg, mean weight 51.1 ± 0.55 kg) ($n = 19$) were obtained from a commercial source. The animals were housed individually in straw lined pens, and were allowed food (standard pig diet) and water *ad libitum* for 5 days prior to the experiment. From 12 hours prior to the experiment access to food was withdrawn though water remained freely available.

2.2 Surgical procedure

Animals were premedicated with midazolam hydrochloride ("Hypnovel", Roche Products Ltd, UK) by intramuscular injection (0.6 mg.kg^{-1}), and anesthesia induced by inhalation of 5 % isoflurane ("IsoFlo", Abbott Laboratories, UK) in 70 % oxygen and 30 % nitrous oxide. The animals were intubated with a size 10 endotracheal (ET) tube which had been lined with polytetrafluoroethylene (PTFE) as previously described (Fairhall *et al.*, 2008). Surgery was performed under aseptic conditions by cut down procedure for insertion of arterial and venous catheters. A 'Pulse Contour Continuous Cardiac Output' (PiCCO) catheter was inserted into the right femoral artery and attached to a PiCCO monitor, allowing continuous measurement of cardiac output, systemic artery pressure, stroke volume, and core temperature. Periodic thermal dilution analysis allowed estimation of extravascular lung water. Other non invasive physiological monitoring devices were attached to a Propaq 106EL monitor. In the pig the presence of a sub-urethral diverticulum makes urethral catheterization difficult and, therefore, a Bonanno catheter was introduced into the bladder via an open cystotomy. Throughout the study a maintenance infusion of 0.9% w/v sodium chloride and 4% w/v glucose ($2.5 \text{ ml.kg}^{-1}.\text{h}^{-1}$) was delivered to replace insensible losses.

Once surgery was completed, intravenous anesthesia was established and maintained using fresenius propoven 1% ($10 - 12 \text{ mg.kg.h}^{-1}$) (Propofol® Fresenius Kabi Ltd, Cheshire, UK) and alfentanil hydrochloride ($0.5 - 2.5 \text{ µg.kg.h}^{-1}$) (Rapifen® Janssen-Cilag Ltd, McGregor Cory Ltd, UK).

2.2.1 Exposure to HD

The system for exposing the animals to HD has been described in detail previously (Fairhall *et al.*, 2008). In brief, the animals were maintained supine whilst breathing spontaneously and baseline measurements were taken for 1 hour. They were then connected to a dynamic exposure apparatus via a PTFE-lined ET tube, a size 2 Fleisch pneumotachograph and a sample port for 10 min. HD vapor was generated from two custom 'Porton' vapor generators (PVGs) operated in parallel. The apparatus was set up to deliver the required inhaled dose over a ten minute exposure.

HD concentration was measured in real time using a Total Volatile Organic Compound photoionisation detector (TVOC PID) (Ion science, Royston, UK) which gave an approximate concentration and also by sampling the vapor during exposure at a constant rate onto porapak absorption tubes for accurate off-line analysis. These were then analyzed by solvent (hexane) elution and quantitative gas chromatography-flame photometric detection (GC-FPD) in a UKAS accredited laboratory. For this a mid-polar capillary column was used and the mobile phase was helium carrier gas at a flow rate of 2.5 ml.min⁻¹.

For the exposures a specific inhaled dose was targeted for each of the three HD groups. This was calculated using concentration, time, the animal's body weight and total inhaled volume¹. The concentration of HD generated for each group was kept constant and the desired inhaled dose reached by varying the length of exposure. Concentration was kept constant as the PVGs are slow to equilibrate following a temperature change and therefore not practicable to keep changing during an exposure. Effort was also made to maintain the animals' minute volumes between 6 and 8 l.min⁻¹ by controlling the dose of anesthetic. The inhaled dose was then estimated every minute during the exposure, and the exposure terminated when the inhaled dose reached the desired value. Thus, whilst the exposure duration was planned to be 10 minutes, in practice it varied between 6.5 and 12.6 minutes to account for uncontrollable variations in the animal's minute volume from the 'ideal' 7 l.min⁻¹.

2.2.2 6 hour study

Animals were randomly assigned to either air control (n = 4) or mustard exposed groups - 60 µg.kg⁻¹ HD (low, n = 5), 100 µg.kg⁻¹ HD (medium, n = 5) or 150 µg.kg⁻¹ (high, n = 5).

2.2.3 12 hour study

Animals were randomly assigned to either air control (n = 6) or HD exposed groups - 60 µg.kg⁻¹ HD (low, n = 3) or 100 µg.kg⁻¹ HD (medium, n = 7).

2.2.4 Physiological measurements

Cardiac and pulmonary physiological parameters were recorded every five minutes for the first 30 minutes following exposure and then every 30 minutes throughout the course of the experiment (up to 6 or 12 hours). Derived variables were calculated using standard formulae (Edwards *et al.*, 1993).

Arterial and mixed venous blood samples were taken prior to exposure, immediately post exposure, at 30 minutes post exposure and then hourly post exposure for the duration of the experiment. At each time point an aliquot of arterial blood was placed into a heparinized blood gas syringe (Sims Portex Ltd, UK) and immediately analyzed using a GEM Premier 3000 blood-gas analyzer (Instrumentation Laboratories UK Ltd, Cheshire, UK). A second aliquot of arterial blood was mixed and refrigerated for later hematological analysis. A third sample was separated into appropriate anticoagulants, centrifuged (3000 rpm, 10 min) and the plasma stored at -80 °C for analysis. In addition

¹ Inhaled Dose (µg.kg⁻¹) = HD Concentration (µg.l⁻¹) x (total volume breathed during exposure (litres)) / Body Weight (kg).

to the collection of blood, total urine output was recorded hourly and 12 ml aliquot samples retained (-80 °C).

In the 12 hour study a suctioning protocol was implemented (with Customer agreement) as a result of two early deaths due to blockage of the ET tube. From 6 hours post exposure the ET tube was suctioned at hourly intervals, with the suction line restricted so that it could not pass the end of the ET tube and suction secretions from the lungs.

Respiratory parameters were recorded in real time using eDAcq (**EMMS Data Acquisition**) software (Infodisp, UK). Minute and tidal volume, lung resistance and compliance, along with other lung function variables, were monitored in real time throughout, and subsequent to, the exposure.

2.3 ***Post mortem and histopathology***

At the end of the 6 or 12 hour monitoring period, or when the animal became moribund (defined as asystole and a mixed venous oxygenation (SvO₂) of less than 15%) the animal was culled by an intravenous overdose of sodium pentobarbitone, 200 mg.ml⁻¹ (“Euthatal” Rhone Merieux Ltd., Harlow, Essex) and a full *post mortem* (PM) examination carried out. PM was carried out wearing personal S10 respirators to prevent contamination from any potential HD ‘off-gassing’. Following removal of the ET tube the trachea was clamped, the thorax opened and, after the animal was exsanguinated by severing the major arteries, the lungs and heart were removed. A small incision was made in the trachea just below the clamp. A flexible bronchoscope (Fiberoptic Bronchoscope, Olympus BF-4B2) was then inserted and maneuvered into the right median lobe. Aliquots of sterile saline (4 x 40 ml) were expelled into the lobe, the syringe pulled back, and the fluid aspirated between each aliquot. The aliquots were pooled together and placed on ice. Following lavage, the lungs were weighed for lung wet weight/body weight (LWW:BW) ratio determinations (the weight of the remaining lavage saline being taken into account). Lung samples were taken, weighed and then dried in an oven (40° C) until a stable weight had been achieved, to determine lung wet weight:dry weight (LWW:DW) ratios. Samples from each of the lobes, together with samples from other major organs were taken, fixed in neutral buffered formalin and processed for histopathological examination using standard methods. Lung samples stabilized in RNAlater were sent to Dr James Dillman III (US Army Medical Research Institute of Chemical Defense, USAMRICD). These will be analyzed on a porcine Affymetrix gene array chip.

2.4 **Sample Processing and Hematology**

A standard eight test hematological analysis was carried out on arterial blood samples and BALF (4°C) using a Coulter Ac-T 5 diff CP (Beckman Coulter) series analyzer. Differential WBC counts (macrophages, lymphocytes, monocytes, eosinophils, neutrophils and basophils) were obtained manually from blood smears and BALF slides (Shandon Cytospin, 1:5 dilution with 0.9% w/v sodium chloride, 1800 rpm, 10 mins) stained with DifQuik, using a Zeiss Axioskop 40 microscope. The remaining BALF was spun (10 min, 3000 rpm) and the supernatant used to determine protein content (Coomassie blue; Bradford, 1976). 20 ml of supernatant was stored (-80°C) for future

analysis of inflammatory mediators. Mediators were measured using commercially available standard quantitative porcine ELISA kits (R&D Systems, Abingdon, UK). Analysis of desmosine, collagenase activity, elastase activity and myeloperoxidase activity was carried out at the University of Portsmouth using commercially available reagents (report Annex A). Clinically relevant biochemical parameters e.g. albumin levels, were measured on an IL-300 chemistry analyzer (Instrumentation Laboratory, UK) using standard techniques (Annex B).

Urine samples were taken for the analysis of sulfur mustard metabolites, of specific interest were the mono-sulfoxide β -lyase metabolite 1-methylsulfinyl-2-[2(methylthio)ethylsulfonyl]ethane] (MSMTESE) and bis-sulfoxide metabolite 1,1'-sulfonylbis[2-(methylsulfinyl)ethane] (SBMSE), which are structures 14 and 15 respectively in Figure 1 (Black *et al.*, 1992). Urine samples were analyzed for MSMTESE using the method published by Read RW and Black RM (2004), modified by use of an alternative C₁₈ Liquid Chromatography (LC) column (ACE 3 C18HL, 150 mm x 2.1 mm). Calibration was performed using urine (from pigs exposed to air) that had been spiked with MSMTESE over the range 0.1 to 50 ng.ml⁻¹. Deuterated (D₆) MSMTESE was used as internal standard for quantization.

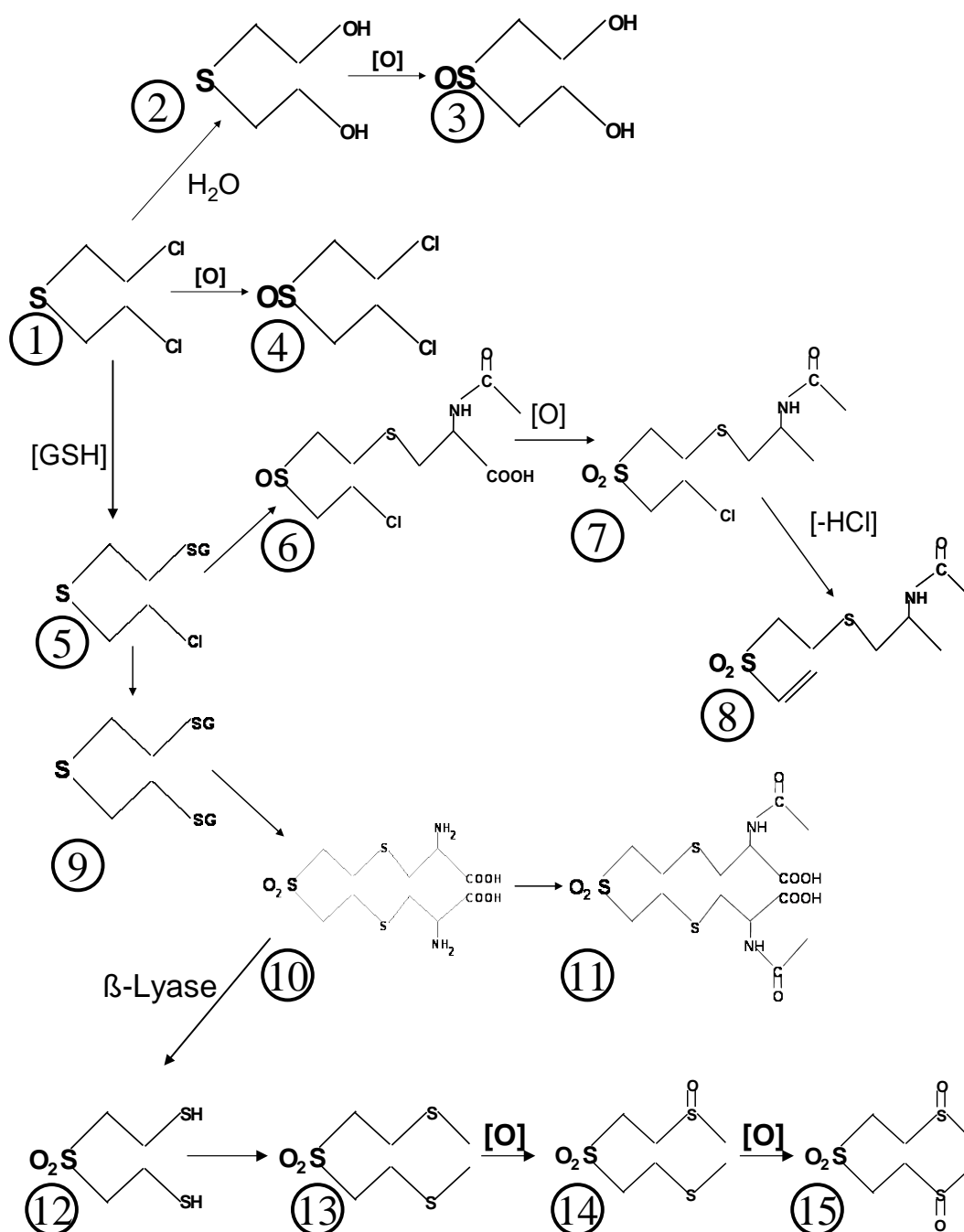


Figure 1 - Proposed metabolic fate of HD (adapted from (Black *et. al.*, 1992)).

Key [GSH] = glutathione; SG = glutathionyl. Metabolites are 1: Sulphur mustard (HD); 2: Thiodiglycol; 3: thiodiglycol sulfoxide; 4: mustard sulfoxide; 5: mono-glutathione-HD adduct; 6: 1-[S-(N-acetylcysteinyl)]-2-(2-chloroethylsulphanyl)ethane; 7: 1-[S-(N-acetylcysteinyl)]-2-(2-chloroethylsulphonyl)ethane; 8: 1-[S-(N-acetylcysteinyl)]-2-(2-chloroethylsulphonyl)ethene; 9: di-glutathione-SM adduct; 10: 1,1'-sulphonylbis[2-S-cysteinyl]ethane; 11: 1,1'-sulphonylbis[2-S-(N-acetylcysteinyl)ethane]; 12: 1,1'-sulphonylbis(2-thioethane); 13: 1,1'-sulphonylbis[2-(methylthio)ethane]; 14: 1-methylsulphanyl-2-[2-(methylthio)ethylsulphonyl]ethane; 15: 1,1'-sulphonylbis[2-methylsulphanyl]ethane].

2.5 Statistical Analysis

2.5.1 6 hour study

Samples were taken at three points pre-exposure. The values obtained at these time-points were averaged and are presented here as a single baseline data point (t=0). The data for each recorded parameter were grouped into one of two categories; 0 - 3 hours and 3 - 6 hours and were checked for normality using an Anderson-Darling test. Data that was not normally distributed was log transformed where possible. The area under the curve (AUC) was then calculated for each parameter within each category.

Comparisons of the AUC were then made between the control group and HD treatment groups (low, medium, high) using either a 2-sample t-test or, for non-parametric data, a Mann-Whitney U-test. Data were expressed as mean (\pm SEM) and p values <0.05 were considered significant. For statistical analysis of inhaled dose, cytokine and MSMTESE levels, cell differentials and LWW:BW and LWW:DW ratio determinations, Bartlett's test was used to assess whether equal variance could be assumed and normality assessed using normal probability plots. Two sample t-tests and analysis of variance (ANOVA) were then carried out. For non-parametric data, Kruskal-Wallis tests were adopted (Annex C).

2.5.2 12 hour study

Data are expressed as mean \pm SEM. Air controls n = 6. HD (medium) exposed n = 7. Bartlett's test was used to determine whether the variability of the data was consistent between the air controls and HD exposed groups as well as whether it was consistent over time. The data were investigated at different time points (3, 6, 9 and 12 hours post exposure) to test for differences between groups. Normal probability plots were used to decide whether the assumption of a Normal Distribution, and hence a parametric test, was appropriate for the data. A two-sample t-test was used to compare the average data values for the two groups (Annex D).

3 Results

3.1 Survival

In the 6 hour study all air control animals and all animals exposed to low or medium dose HD vapor survived to 6 hours. In the high dose group four animals survived to the end of the study with 2 animals dying within approximately 30 minutes of the 6 hour time point.

In the 12 hour study all air control animals and all animals exposed to low dose HD vapor survived to 12 hours. Three animals died in the medium dose HD group, 2 of which died due to obstructions in the ET tube at 5.5 and 6.5 hours. The third animal died at 10.5 hours from HD poisoning.

3.2 HD Exposures

Figure 2a shows that the target and achieved inhaled doses for each of the dosing groups in the 6 hour study were not significantly different. Achieved mean inhaled doses were 67.2 ± 4.3 , 100.6 ± 4.6 and $157.8 \pm 10.0 \mu\text{g.kg}^{-1}$ for the low, medium and high groups respectively ($p = 0.17$, 0.90 and 0.48 compared to target equivalent). This demonstrates that the exposure system could be successfully used to control the inhaled dose as desired. The three achieved mean inhaled dose groups were significantly different from each other ($p < 0.001$).

Figure 2b shows that the target and achieved inhaled doses for each of the dosing groups in the 12 hour study were not significantly different. Achieved mean inhaled doses were $66.5 \pm 6.1 \mu\text{g.kg}^{-1}$ for the low group and $101.0 \pm 3.9 \mu\text{g.kg}^{-1}$ for medium group ($p = 0.40$, and 0.80 compared to target equivalent). The achieved mean inhaled dose groups were significantly different from each other ($p < 0.001$).

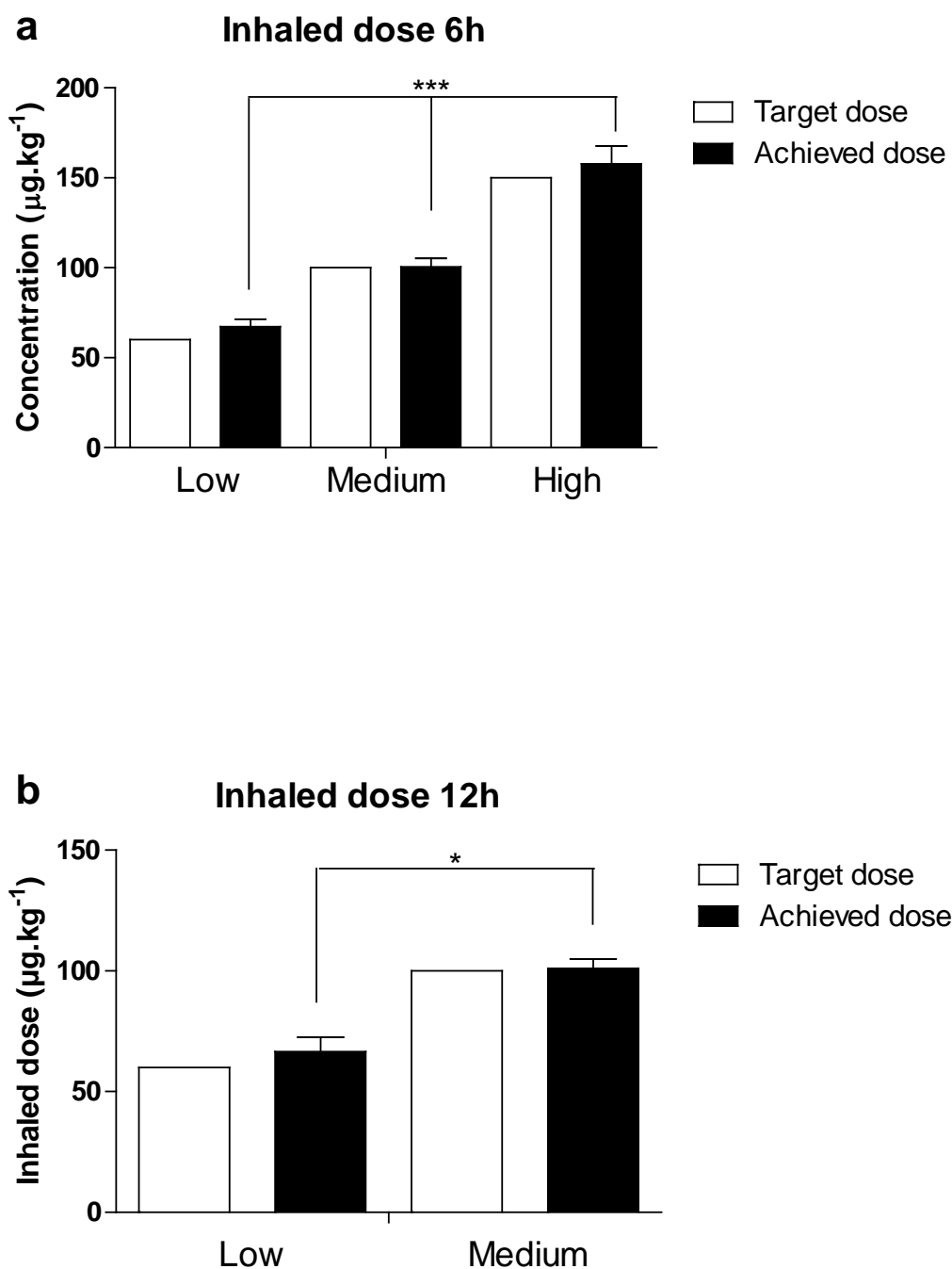


Figure 2 - Target versus achieved doses for each of the HD exposed groups. Data are expressed as mean \pm SE. Graph "a" shows the results from the 6 hour study ($n = 5$), ***significant difference between each of the HD dose groups ($p < 0.001$). Graph "b" shows the results from the 12 hour study ($n = 3$ Low and $n = 7$ Medium), * significant difference between the HD groups ($p = 0.0012$).

3.3 Physiology and hematology

In both the 6 and 12 hour studies air control animals maintained arterial oxygenation throughout. In the 6 hour study there was a significant decrease in oxygenation from 3 - 6 hours in both the medium (* $p = 0.035$) and high ([#] $p = 0.020$) dose HD exposed groups when compared to the air control group (Figure 3a).

In the 12 hour study there was a significant decrease in oxygenation at 6, 9 and 12 hours in the medium ($p < 0.05$) dose HD group when compared to air control group (Figure 3b).

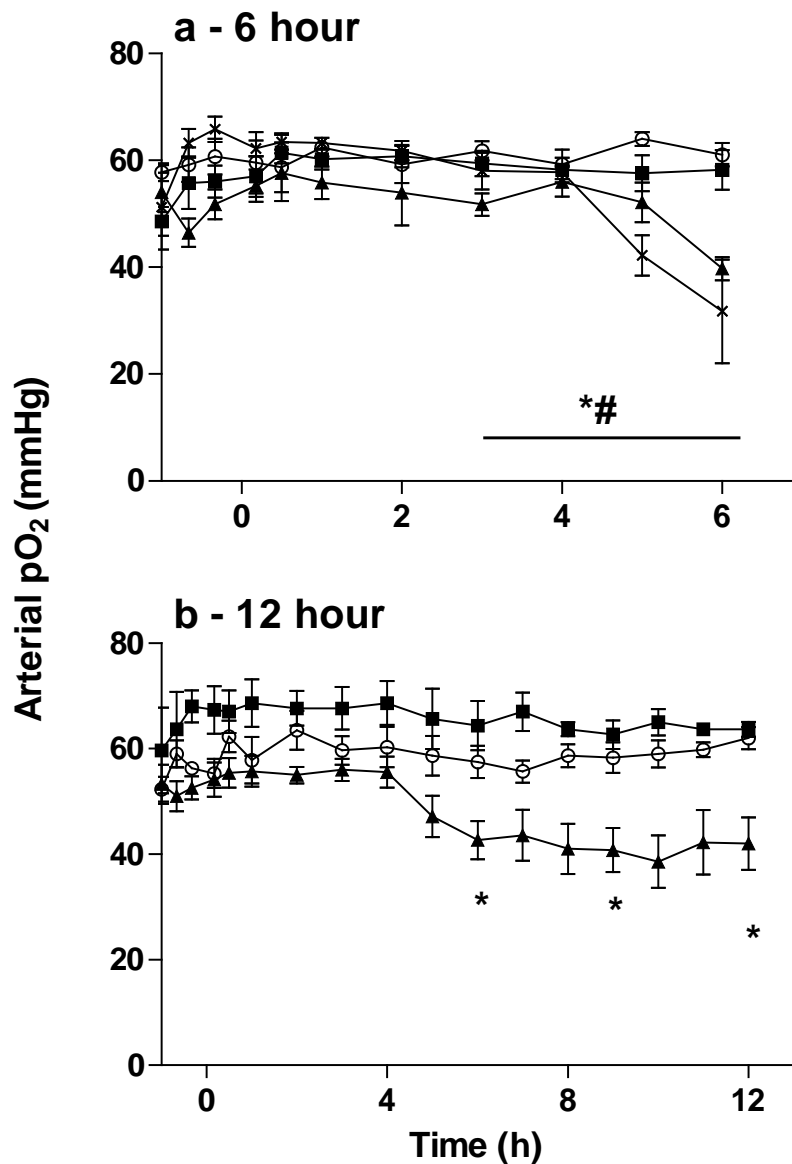


Figure 3 - Changes in arterial blood oxygen tension following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE. Graph 'a': *significant difference between air and medium dose HD group over 3-6 h ($p < 0.05$). [#]Significant difference between air and high dose HD group over 3 - 6 h ($p < 0.05$) in the 6h study. Graph 'b': *significant difference between air and the medium HD group at 6, 9 and 12h ($p < 0.05$).

In both the 6 and 12 hour studies air control animals maintained oxyhemoglobin levels throughout. At 3 - 6 hours post exposure there were significant dose dependent decreases in oxyhemoglobin levels when compared to air controls (air v medium * $p = 0.03$; air v high # $p = 0.02$) in the 6 hour study (Figure 4a). In the 12 hour study there were significant decreases in the medium (p<0.05) dose HD group at 6, 9 and 12 hours post exposure when compared to the air control group (Figure 4b).

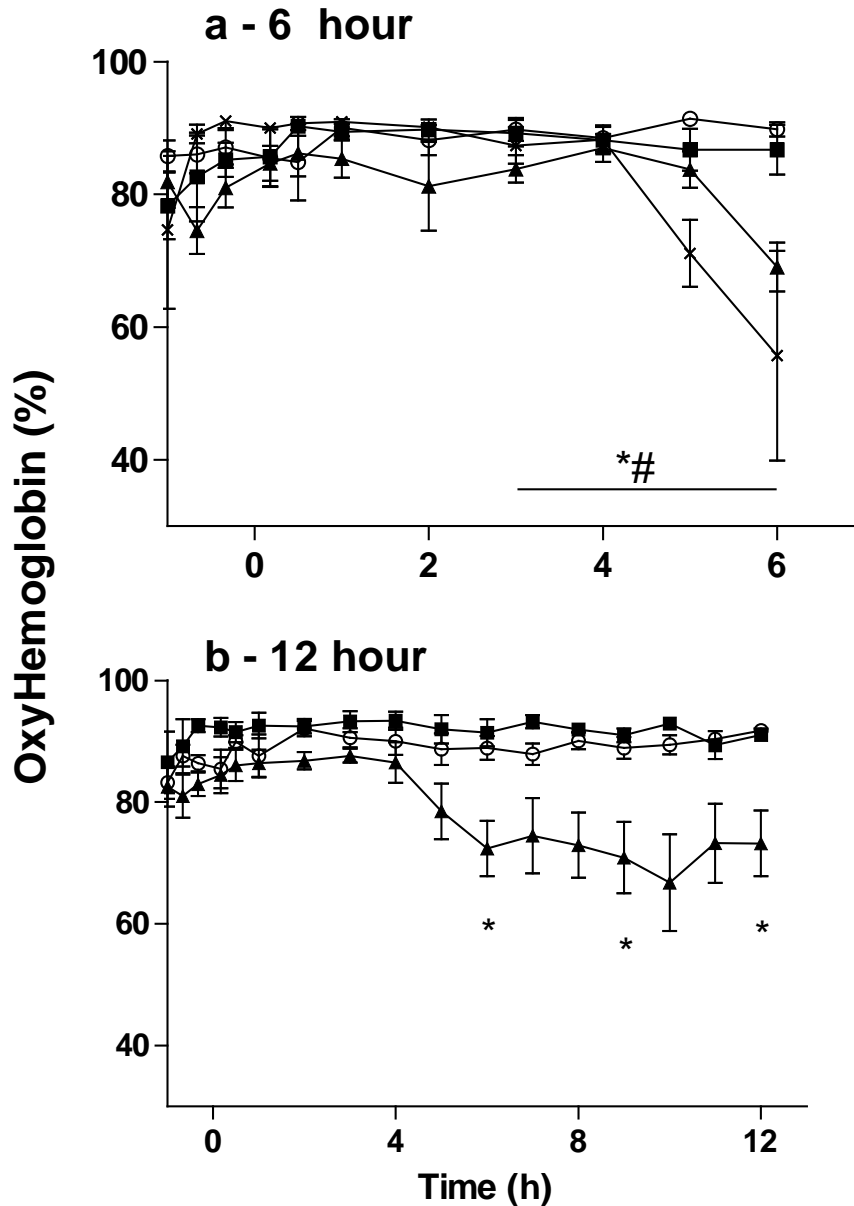


Figure 4 - Changes in oxyhemoglobin concentration following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE. Graph "a": *Significant difference between air and medium dose HD group over 3-6 h ($p < 0.05$). #Significant difference between air and high dose HD group over 3 - 6 h ($p < 0.05$) in the 6h study. Graph "b": *significant differences between air and medium dose HD group at 6, 9 and 12h ($p < 0.05$).

Figure 5 shows changes in shunt fraction, the proportion of blood passing through the lungs which remains un-oxygenated due to perfusion and ventilation mismatch. The shunt fraction for air control animals in both the 6 and 12 hour studies was maintained at normal baseline levels. The shunt fraction of all HD exposed groups was significantly higher than the air control group from 3 - 6 hours (air v low $^{\dagger}p = 0.014$; air v medium $^*p = 0.003$; air v high $^{\#}p = 0.047$) in the 6 hour study (Figure 5a). In the 12 hour study the shunt fraction of the medium dose HD animals was significantly increased at 6 hours post exposure ($^*p = 0.047$) (Figure 5b).

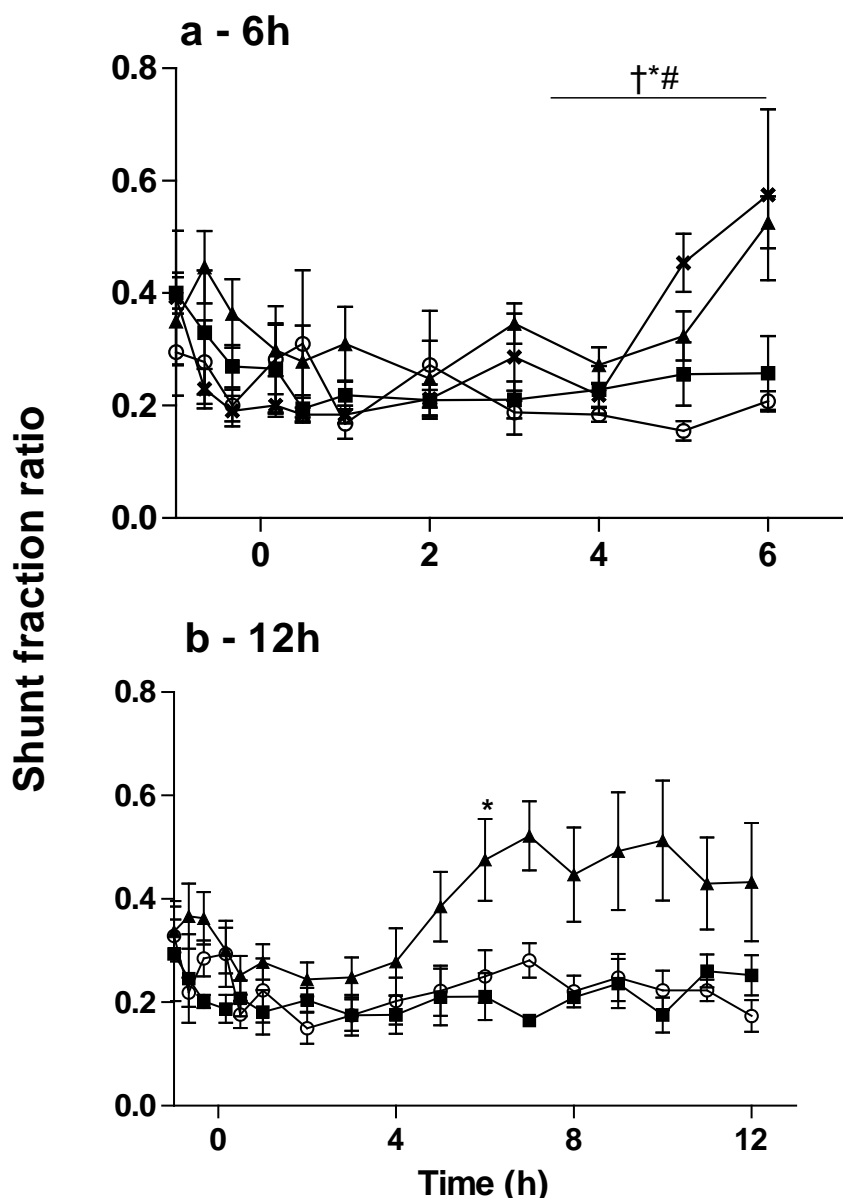


Figure 5 - Changes in shunt fraction (Q_s/Q_t) following exposure to low (■), medium (▲) or high (x) doses of HD or exposure to air (○). Data are expressed as mean \pm SE. Graph 'a': † Significant difference between air controls and low dose HD group over 3 - 6 h ($p < 0.05$). * Significant difference between air controls and medium dose HD group over 3 - 6 h ($p < 0.05$). $^{\#}$ Significant difference between air controls and high dose HD group over 3 - 6 h ($p < 0.05$) in the 6h study. Graph 'b': * significant difference between air controls and medium dose HD group at 6h ($p < 0.05$)

Figure 6a shows a statistically significant decrease in the arterial base excess of the high dose HD exposed group when compared to air controls from 3 - 6 hours in the 6 hour study ([#]p = 0.021). Figure 6b shows a statistically significant decrease in the arterial base excess of the medium dose HD group at 3, 6, 9 and 12 hours when compared to air controls (*p<0.05). There was also a statistically significant decrease in arterial blood bicarbonate levels in HD exposed animals when compared to air controls 0 - 3 hours post exposure (air v low [†]p = 0.028; air v medium *p = 0.016; air v high [#]p = 0.006) (Figure 7a). The 12 hour study showed a significant decrease in the bicarbonate levels in the medium dose HD group at 3, 6, 9 and 12 hours post exposure when compared to air controls (*p<0.05) (Figure 7b). This was not reflected by a change in the pH of any of the HD exposed animals, which remained stable throughout the 6 and 12 hour studies and were not statistically significantly different from air controls (Annex G).

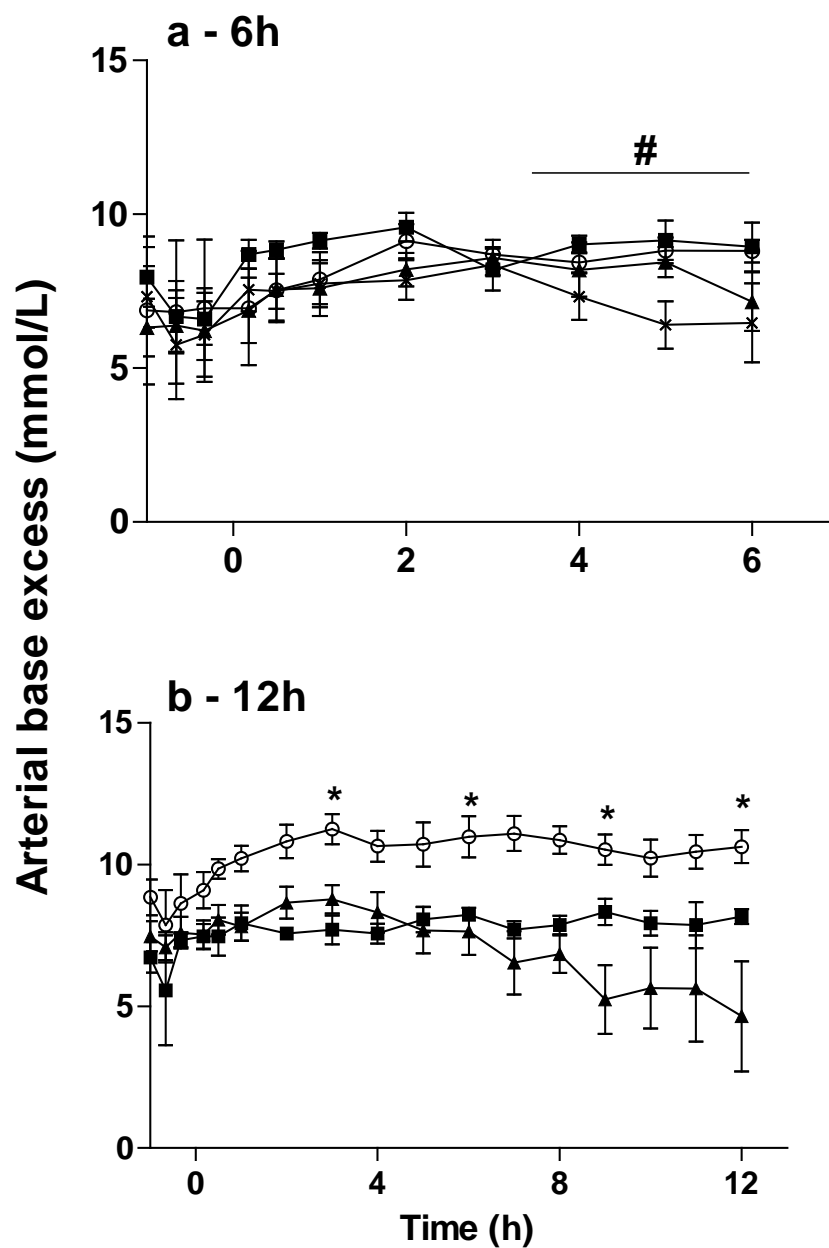


Figure 6 - Changes in arterial base excess following exposure to low (■), medium (▲) or high (x) doses of HD or exposure to air (o). Data are expressed as mean \pm SE. Graph 'a': # Significant difference between air controls and high dose HD group over 3 - 6 h ($p < 0.05$) in the 6h study. Graph 'b': *significant differences between air controls and the medium dose HD group at 3, 6, 9 and 12h ($p < 0.05$) in the 12h study.

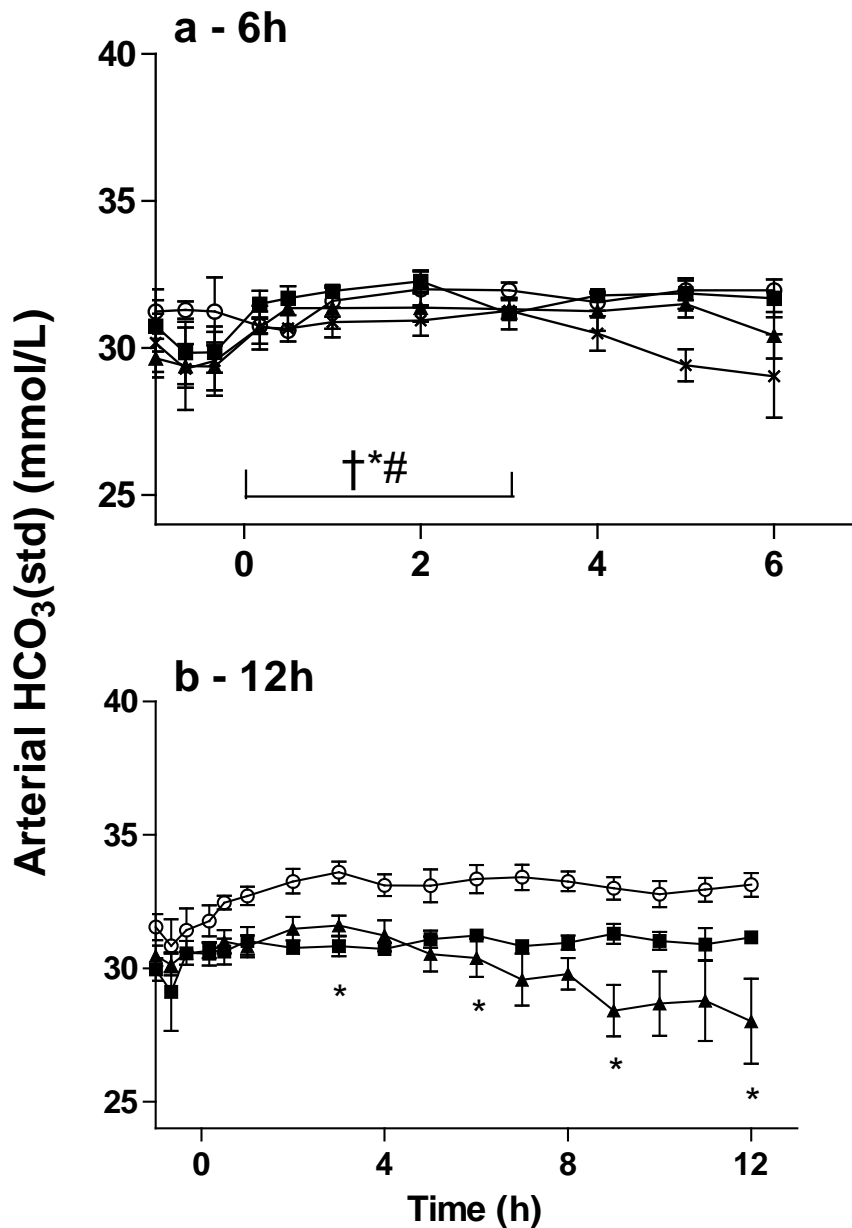


Figure 7 - Changes in levels of arterial blood bicarbonate concentration following exposure to low (■), medium (▲) or high (x) doses of HD or exposure to air (o). Data are expressed as mean \pm SE. Graph 'a': significant differences in arterial bicarbonate in all HD groups 0 - 3h compared to air controls. Graph 'b': *significant difference in arterial bicarbonate at 3, 6, 9 and 12h ($p < 0.05$).

Of the blood gas parameters investigated there were no significant differences between HD exposed groups and air controls over the 6 hour study (Annex G). However in the 12 hour study there were significant reductions in arterial blood sodium concentration at 3, 6, 9 and 12 hours post exposure in the medium dose HD group when compared to air controls ($*p < 0.05$) (Figure 8b). There were also significant increases in hematocrit in the medium group at 9 and 12 hours post exposure when compared to air controls ($*p < 0.05$) (Figure 9b).

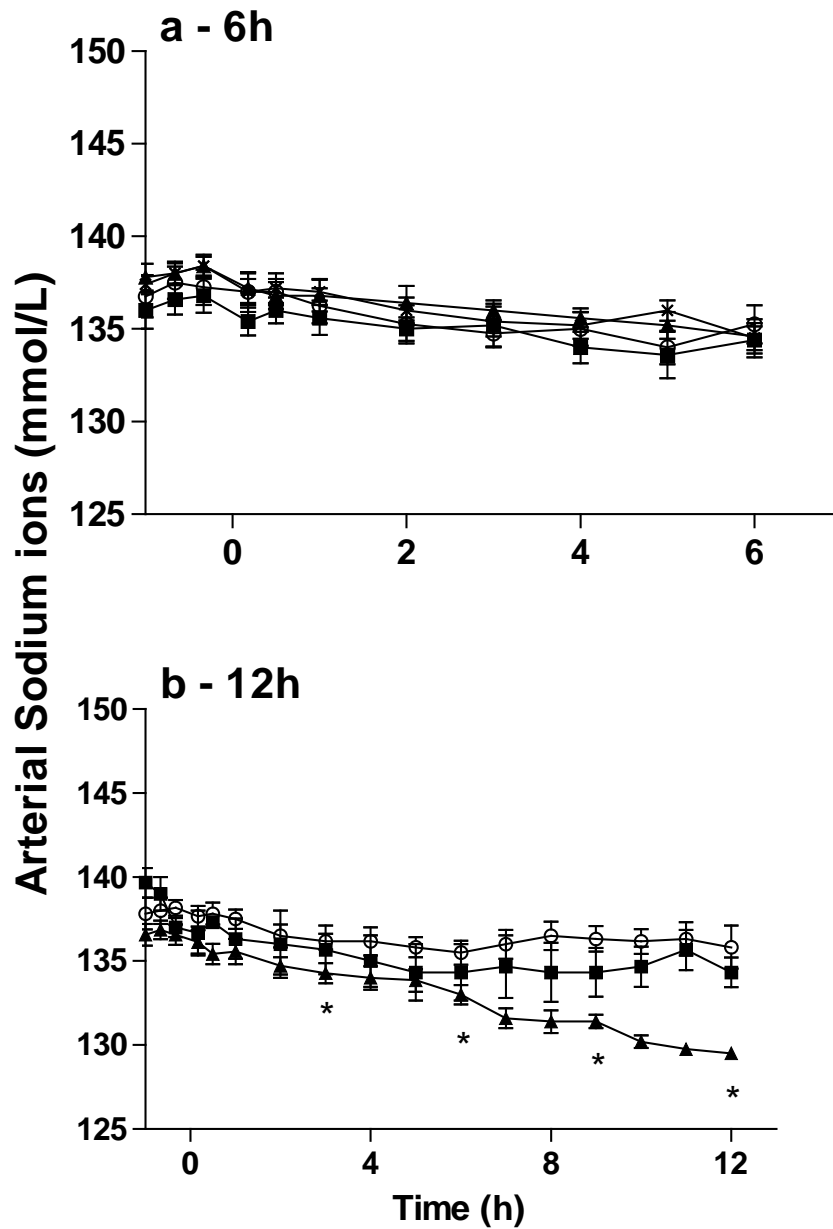


Figure 8 - Changes in levels of arterial blood sodium concentration following exposure to low (■), medium (▲) or high (x) doses of HD or exposure to air (o). Data are expressed as mean \pm SE. Graph 'a': no differences in arterial sodium up to 6h. Graph 'b': *significant difference in arterial sodium at 3, 6, 9 and 12h ($p < 0.05$).

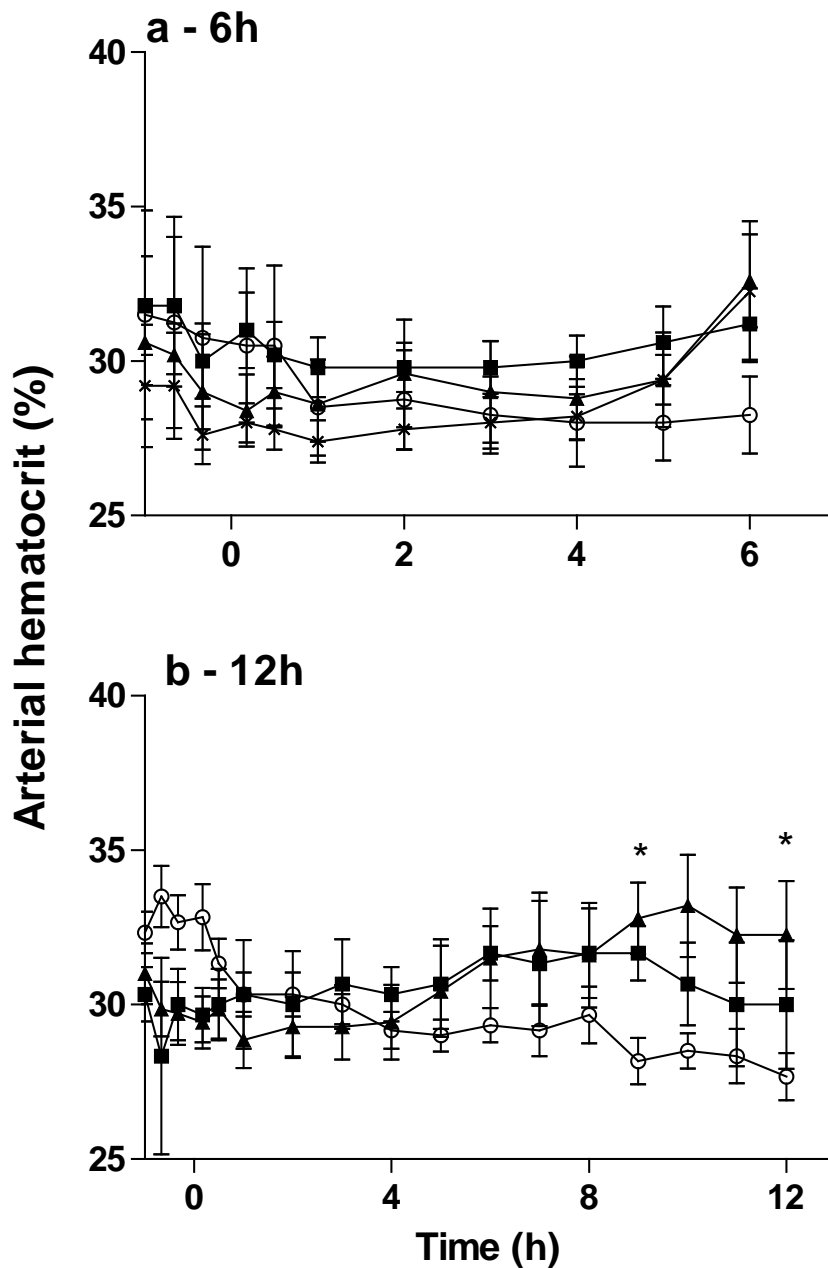


Figure 9 - Changes in arterial blood hematocrit following exposure to low (■), medium (▲) or high (x) doses of HD or exposure to air (o). Data are expressed as mean \pm SE. Graph 'a': no differences in arterial hematocrit up to 6h. Graph 'b': *significant difference in arterial hematocrit at 9 and 12h ($p < 0.05$).

3.4 Portsmouth Sample analysis (12 hour study)

The results show a significant increase in urinary, but not terminal BALF, desmosine levels post HD challenge, while there were no significant effects in air controls. BALF desmosine concentrations correlated significantly with BALF neutrophil numbers, both as a percentage of total cells and absolute numbers of neutrophils. There was no

correlation in urinary desmosine at any time point with BALF biomarkers (neutrophil numbers, IL-8, neutrophil elastase, myeloperoxidase (MPO) or matrix metalloproteinase (MMP)) or plasma MMP activity in the HD challenged group. There were no significant differences in BALF MMP activity, MPO activity or in elastase activity, although trends towards an increase in elastase and MPO activity (markers of neutrophil activation) can be seen between groups. Plasma collagenase activity did not differ following exposure to HD at any time point or following sham challenge (Annex A).

3.5 Urine Adducts

MSMTESE is not a naturally occurring molecule and is an unequivocal marker of exposure to HD. There was an increase in MSMTESE levels seen over the course of the 6 hour study in all HD exposed groups. Levels of metabolite of approximately 0.2 ng.ml^{-1} were detected as early as 2 hours post exposure in the high dose HD exposed group (Figure 10a). The apparent peak at 5 hours post exposure in the high dose HD exposed group results from an individual animal that died shortly after this sample had been taken. This one increased data point (5.2 ng.ml^{-1} for this individual) accounts for the larger variation seen within this group at this time point. In the 12 hour study levels of MSMTESE were also seen at 2 - 4 hours with large increases seen from 6 hours onwards (Figure 10b).

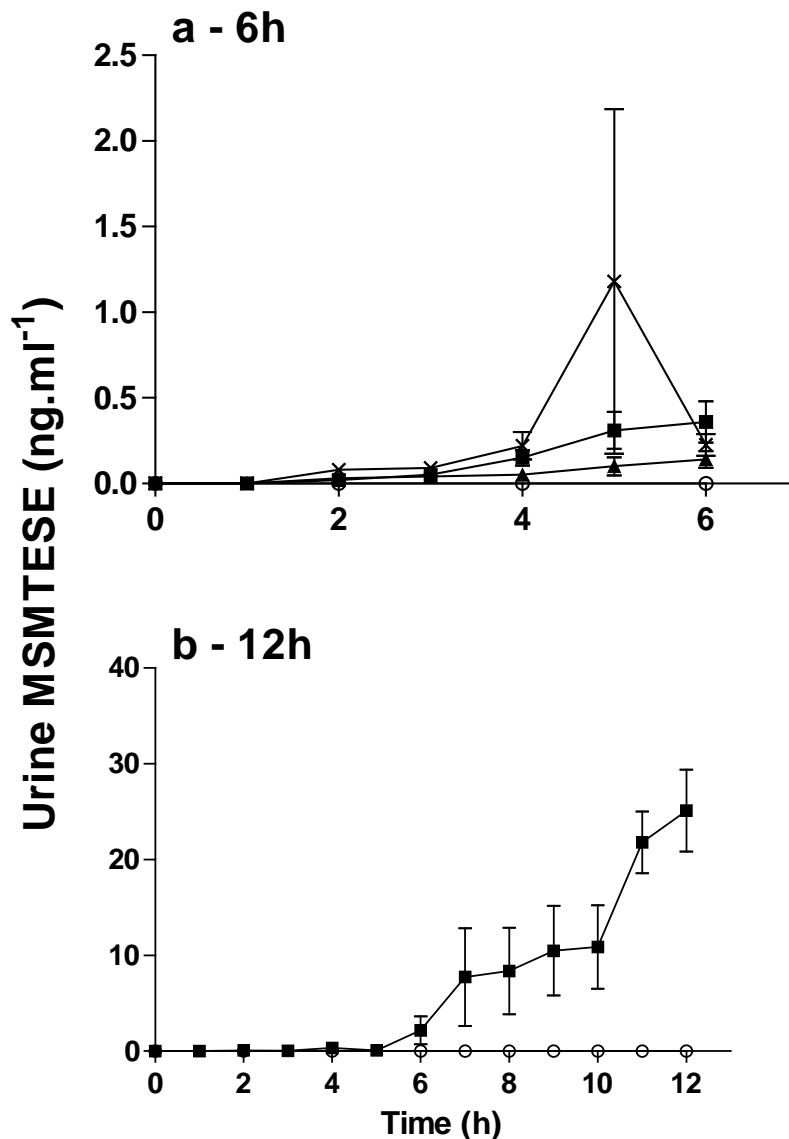


Figure 10 - Concentrations of the mono-sulfoxide β -lyase metabolite 1-methylsulfinyl-2-[2(methylthio)ethylsulfonyl]ethane (MSMTESE), in the urine following exposure to low (■), medium (▲), or high (x) doses of HD, or exposure to air (○). Data are expressed as mean \pm SE. Graph 'a': 6h study urine MSMTESE concentration. Graph 'b': 12h study urine MSMTESE concentration.

3.6 Histopathology.

Pathology samples were sent to the Veterinary Laboratories Agency for an independent pathologist to score the slides for pathological changes. A scoring system was developed to assess the lung pathology, based on: bronchiolar epithelial degeneration and necrosis; hypertrophy/hyperplasia of bronchiolar epithelium; cell debris in the bronchiolar lumen; perivascular (and peribronchiolar) edema, fibrin and cell infiltrates; alveolar edema and fibrin; alveolar cell infiltrates; interstitial (septal) edema; hemorrhages. Parameters were evaluated as follows: 0: Not observed; 1: Mild; 2: Moderate; 3: Severe. The full reports,

including injury scoring, are contained in Annex E (6 hour report) and Annex F (12 hour report).

Examination of the tracheal epithelium in the 6 hour study revealed a dose dependent increase in severity of HD induced damage (Figure 11). Air control trachea (A) showed intact ciliated tracheal epithelium and normal lamina propria and submucosal structures. Exposure to both low dose (B) and medium dose HD (C) gave rise to edema in the lamina propria and mild inflammatory cell (neutrophil) infiltration throughout the mucosa and submucosa. Infiltration was more marked in the medium dose group. Both groups had apparent blood vessel congestion and areas of nuclear pyknosis. In addition to the above reported effects, exposure to high dose HD (D) also caused localized sloughing and loss of tracheal epithelium. Damage within the lower parts of the airways was found to be mild. Most relevant was the necrosis of the bronchiolar epithelium, the presence of cellular debris in the bronchiolar region and evidence of septal and perivascular edema. Plugs of inflammatory cells were found throughout and were found to consist mostly of neutrophils. Pathological assessment in the 12 hour study showed intact ciliated tracheal epithelium and normal lamina propria and submucosal structures in the air control group. Areas of epithelial necrosis, septal and perivascular edema and cell debris in the lumen were identified in the HD group at 12 hours, which shows a progression of injury to 12 hours in the medium dose HD group compared to the 6 hour study (Figures 12 and 13).

All data collected during this study which has not been reported in the main body of the text is captured in Annex G.

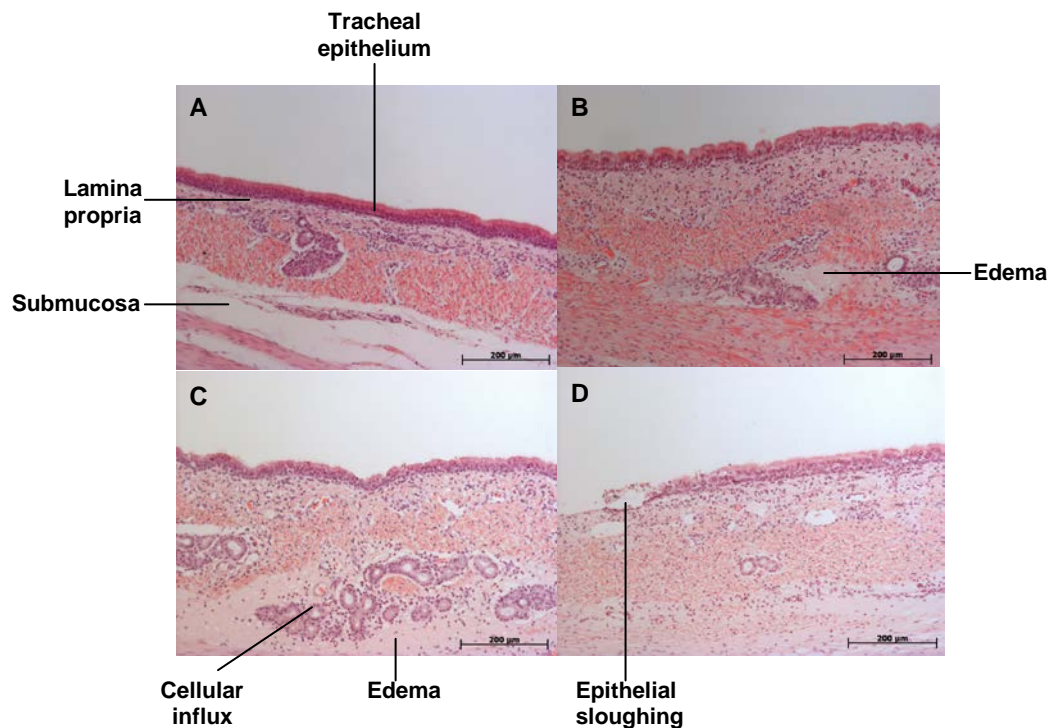


Figure 11 - Tracheal epithelium following exposure to HD (original magnification x 100). A) Air control, B) Low dose HD, C) Medium Dose HD and D) High dose HD in the 6h study.

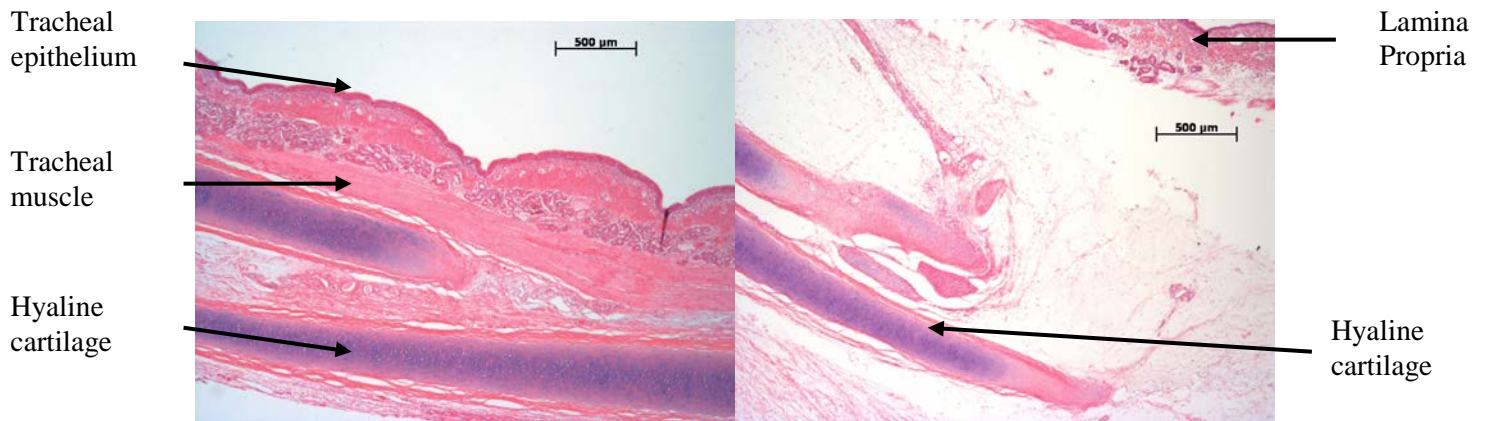


Figure 12 - Transverse section through the trachea of an air control (left) and HD (right) exposed animal (x25).

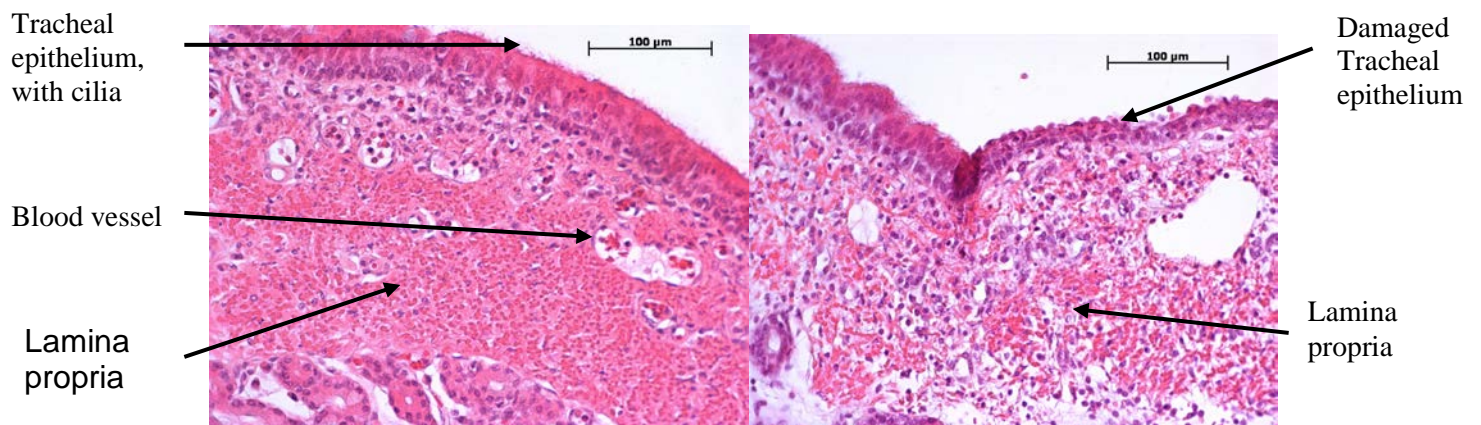


Figure 13 - High power image of transverse section through the trachea of an air (left) control and HD (right) exposed animal (x200)

4 Discussion

The mechanism by which HD acts in the lung remains poorly understood. Since the majority of deaths associated with HD exposure have a degree of pulmonary involvement there is a requirement to further elucidate the underlying mechanisms of HD induced ALI. This report details the development of a porcine model of inhaled HD ALI, in order to enable the future assessment of therapeutic candidates.

The aim of the 6 hour study was to investigate the effects of a range of doses of HD vapor exposure to the lung. Using a novel exposure system (previously described) the lung was exposed directly to HD vapor, producing a highly consistent, reproducible exposure with a high degree of accuracy in attaining the target inhaled doses. This study demonstrated stable physiology and pathology in air control animals over 6 hours, giving confidence in the chosen model. From the 6 hour study two inhaled doses (low and medium) were identified to take forward into the 12 hour study. The physiological condition of the animals from the high dose HD group at 6 hours supports the opinion that animals exposed to this concentration would not have survived out to 12 hours and that the severity of the injury was too great given that two of the animals exposed to this dose died before 6 hours.

The aim of the 12 hour study was to investigate the longer term effects of inhaled HD and establish a model suitable for investigating treatments. The 12 hour study demonstrated stable physiology and pathology in air control animals. The low dose HD group was rejected after 3 animals were exposed as the degree of injury was not significantly different from air controls in terms of oxygenation and pathology. Therefore the medium inhaled dose of HD was used in the full 12 hour study.

Physiological, biochemical and pathological parameters were affected by the inhalation of HD. Dose-dependent changes were observed in the 6 hour study; these were initially mirrored and then continued to develop in the 12 hour study. Following exposure to inhaled HD there was a symptom free period with little change occurring in any of the physiological parameters measured before 4 hours post exposure. This is in agreement with studies that have reported the effects of inhaled HD poisoning in man (Balali-Mood and Hefazi, 2006; Papirmeister, 1991).

Exposure to increasing doses of HD, in a 6 hour period, and increasing the timeframe of investigation to 12 hours, resulted in a number of physiological parameters changing as the lung injury developed. Increasing doses of HD resulted in decreases in arterial blood oxygenation and oxyhemoglobin levels, as well as an increase in shunt fraction. HD exposure caused dose dependent damage to the lung, including sloughing of the tracheal epithelium. It is hypothesized that the cell debris produced travels deeper into the airways blocking smaller bronchi. Consequently, this causes impaired gas exchange creating a mismatch between ventilation of the lungs and perfusion, resulting in an increase in the proportion of un-oxygenated blood traveling through the lungs. A dose dependent reduction in oxyhemoglobin levels confirmed the increased proportion of un-oxygenated blood, as did the increase in reduced hemoglobin levels (Annex G). Over the 12 hour study the medium dose HD caused significant decreases in arterial blood

oxygenation, oxyhemoglobin and a corresponding increase in reduced hemoglobin levels from 6 to 12 hours, however although the shunt fraction was significantly increased at 6 hours, it did not remain significantly raised at longer time points. Implementation of the suctioning protocol from 6 hours (implemented as a result of the two early deaths from ET-tube blockage in the 12 hour study), may have resulted in improved oxygenation as cell debris trapped in the ET tube was cleared, permitting improved ventilation. However the loss of the two animals prior to the 6 hour time point may have reduced the power of the statistics enough for the analysis to no longer be significant.

In the HD exposed groups significant decreases were also seen in arterial base excess and arterial bicarbonate levels, but not in pH. A decrease in base excess without subsequent change in pH indicates that HD exposed animals displayed signs of respiratory acidosis. It is postulated that as a result of HD exposure the liver may be unable to deal with a surplus of H⁺ ions which bind to bicarbonate ions, forming carbonic acid and in turn CO₂ and water. This will lead to a decrease in the levels of bicarbonate ions (seen as decreasing arterial bicarbonate levels) and hence, base excess. pH is maintained by the renal system. This suggests that the HD exposed animals become acidotic, but not acidemic over the course of both the 6 and 12 hour studies. These results, taken together with the histopathological findings, reflect a similar ALI to that reported in man following inhaled HD exposure. A study of Iranian victims from the Iran-Iraq conflict (Kehe *et al.*, 2009) found significant lung symptoms with 67% of the patients studied having altered blood gas composition. Of these patients 50% were hypoxic and 17% acidotic. This further supports the anesthetized pig as being an appropriate model of inhaled HD poisoning in man. Kehe and Szinicz (2005) also reported the acute effects of moderate HD poisoning in man. Effects include tracheobronchitis, painful and hacking coughing and formation of pseudomembranes. Detachment of the pseudomembranes and resulting obstruction of the lower airways was also reported. In our model, inhalation of HD induces upper airway damage, which is most apparent in the trachea and bronchi. This is supported by the findings of Allon *et al.*, (2009) who found sloughing of the tracheal epithelium, necrosis, edema and blood congestion in the lamina propria in their guinea pig model of inhaled HD induced ALI. Mild lower airway damage was also apparent in our model, the pathology revealing small areas of septal and perivascular edema. Kehe and Szinicz (2005) reported edema in man in both the upper and lower airways following a severe exposure. A lack of change in the LWW:BW and LWW:DW ratios and lavage protein concentration seen at 6 and 12 hours in our HD exposed animals is likely due to the very acute nature of this time course, which is a limitation imposed by use of an anesthetized spontaneously breathing animal model.

A small influx of neutrophils was seen following inhaled HD exposure. Accumulation of neutrophils in air spaces is consistently seen in ALI in both humans and in animal models (Pittet *et al.*, 1997) and it is postulated that they contribute either directly, or indirectly, to lung injury (Wiener-Kronish *et al.*, 1990).

The results of the 12 hour study sample analysis performed at Portsmouth University provide evidence of sulfur mustard-induced neutrophil activation in the lung and elastase-mediated breakdown of the matrix protein elastin, which can be monitored as desmosine in urine. This provides evidence for the presence of activated neutrophils in the lung. Breakdown of lung matrix by MMPs may contribute to sulfur mustard-induced lung injury. This may provide a potential therapeutic option for intervention.

Of interest was the ability to detect MSMTESE at such an early time point post inhaled HD exposure (from 2 hours). MSMTESE and SBMSE are used as diagnostic tools to identify percutaneous exposure to HD, however until now they had not been shown to be produced after inhaled HD exposure. This early production of MSMTESE may provide a valuable diagnostic tool in identifying inhaled HD exposure. MSMTESE, unlike thiodiglycol and its sulfoxide derivative (Balali and Hefazi, 2005), is an unequivocal marker of HD exposure and can be reduced to thioether derivatives for analysis by GC-MS (Black and Read, 1988). Due to large amounts of interfering material extracted from pig urine we were unable to detect SBMSE, this is a limitation in our model and SBMSE may still have potential in diagnosing inhaled HD exposure in humans.

One of the acknowledged limitations of this model is the very acute nature of the study (12 hours), which allows us to investigate the injury in a time frame that may not completely mimic the human time course. For practical reasons, to achieve the chosen inhaled doses a high concentration was delivered over a short exposure duration (approximately 10 minutes). This enabled us to cause an injury within the 6 and 12 hour study periods. This may not reflect the real life situation where man is likely to be exposed to a lower concentration for a longer period of time due to the persistency of HD. Delivery of a high dose over a short period may result in a more severe injury. Secondly, our model was designed to best represent human exposure, bypassing the URT and exposing directly to the lung. This does not completely mimic the real life situation where the nasal passages may absorb some of the HD, however, there is no existing animal model in which this can be achieved. It is possible that the involvement of the URT may remove a percentage of the HD inhaled reducing the dose reaching the lung. This may result in a longer asymptomatic period before an effect on oxygenation, for example, is realized. Despite these differences the changes seen in our model reflect those seen in man but in a condensed timeframe. It will be important to take this into consideration in future studies.

The introduction of a suctioning protocol 6 hours post exposure has addressed one of the limitations of the model, however we acknowledge this is still a concern.

We conclude that the medium dose of inhaled HD used in these studies produces a significant and consistent ALI which is severe but not fatal up to 12 hours and against which the efficacy of candidate therapeutic strategies can be investigated.

It is anticipated that the genomic analysis carried out at USAMRICD will identify specific gene targets to better direct the identification of therapeutic interventions for HD induced lung injury. These therapeutics will then be tested in the 12 hour porcine model of inhaled HD induced ALI to assess their benefits. These therapeutic targets can then be compared with other chemicals causing an ALI e.g. phosgene (Brown et al., 2002), in order to define whether any common pathways exist in the injury process. This might identify new generic candidates for the treatment of chemically induced ALI whose efficacy can then be tested in this robust model.

5 Conclusions and Recommendations

- Exposure to inhaled HD in our terminally anaesthetized spontaneously breathing large white pig model caused significant dose and time dependent changes in physiological parameters from 3-12 hours post exposure.
- Animals were increasingly hypoxic and showed signs of respiratory acidosis.
- Pathology revealed necrosis and erosion of the tracheal epithelium as well as localized sloughing of the epithelium.
- These findings are consistent with those seen in the early stages of ALI and agree with evidence from human exposures, giving us confidence in the applicability of this model in representing human exposure (Balali-Mood and Hefazi, 2006; Papirmeister, 1991).
- Additionally, MSMTESE was detected in the urine from 2 hours post exposure. This could be used as a diagnostic marker of inhaled HD exposure.
- We recommend that our model is an appropriate one in which to investigate the efficacy of promising therapeutic candidates in the treatment of chemically induced ALI.

6 Acknowledgements

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R. Perrott - technical and surgical assistance

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Kay Bridgwater and Ralph Mansson – Statistical analysis

Inhalation team – Exposures

Detection Department – Analytical Chemistry

John Jenner – Technical review

7

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UNCLASSIFIED

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ANNEX A **Report from Portsmouth University: The effects of sulphur mustard on the lungs**

Abstract: Sulfur mustard (HD), infamously known as mustard gas, is a chemical weapon and was an important component of waging war during World War 1 (1914-1918). More recently it has been used as a tool of terror/genocide against Kurdish civilian populations in Iraq (1988). Large stockpiles coupled with easy handling and synthesis make SM an ideal candidate for use in terrorist acts and therefore it is considered a serious threat to world security. Despite extensive research over the past 90 years the exact pathophysiological mechanisms of HD remain elusive. Acute effects of HD are manifested after a dormant asymptomatic period of approximately 3 hours post exposure. HD-induced effects on the respiratory system can be divided into 4 transient phases: 1. the catarrhal state; 2. the pseudomembranous laryngotracheitis state; 3. the pseudomembranous bronchitis and bronchopneumonia state; and 4. the lung abscess and gangrene state². The inflammatory response to HD plays a vital role in the development and exacerbation of symptoms, therefore an understanding of the immunological events elicited by HD would prove to be a key element in terms of prevention and attenuation of HD injury. The investigation aims to determine the role of the neutrophilic response in the lung post HD inhalation, and identification of possible therapeutic targets. Pigs exposed to HD provided a model from which plasma and urine samples could be taken every 2 hours for 12 hours; markers of neutrophil activity were then detected using a competitive ELISA that detects urinary desmosine and a gelatinase/collagenase assay which identifies matrix metalloproteinases (MMPs) present in plasma. Finally, a broncho-alveolar lavage (BAL) obtained post 12 hours exposure was tested for neutrophil activation. A competitive ELISA which detects desmosine, a gelatinase/collagenase assay sensitive to MMPs, a myeloperoxidase assay and a neutrophil elastase assay was used. Significant increases of urinary desmosine was observed post HD exposure implying neutrophil mediated elastase activity in the lung in response to HD.

Hypothesis: Sulfur Mustard induces neutrophil mediated lung elastin breakdown.

Analytical Methods;

A competitive ELISA that detects urinary 'desmosine' and isodesmosine

The following assay reagents were procured from:

Elastin Products company (EPC)
PO BOX 568

Owensville

² Kehe K, Balszuweit F, Emmeler J, Kreppel H, Jochum M and Thiermann H. Sulfur Mustard Research—Strategies for the Development of Improved Medical Therapy. Open access Journal of Plastic Surgery, June 2008.

Missouri 65066

USA

www.elastin.com

The assay was developed by the following key references:

- Laurent, P., et al. (1988). Quantitation of elastin in human urine and pleural mesothelial cell matrix by a sensitive avidin-biotin ELISA for desmosine. J Immunol Methods 1988; 107: 1-11.
- Osakabe, T., et al. Comparison of ELISA and HPLC for the determination of desmosine and isodesmosine in aortic tissue elastin. J Clin Lab analysis 1995; 9: 293-296.
- Cumiskey, W. R., et al. Enrichment and analysis of desmosine and isodesmosine in biological fluids. J Chromatog B 1995; 668: 199-207.

Reagents

1. Desmosine-egg albumin (DEA) conjugate.
2. Desmosine standard.
3. Isodesmosine standard.
4. Rabbit anti-desmosine serum (this antibody has no species specificity but we found it cross reacts with isodesmosine).

Buffers

A1: (veronal buffer-VBS) 0.01M veronal (barbitone), 0.15 M NaCl, 1 mM MgCl₂ and 1 mM CaCl₂. To 100 ml UHQ (ultra high quality) water add 0.1842 g veronal, 0.8766 g NaCl, 0.0203 g MgCl₂·6H₂O, 0.0147 g CaCl₂·2H₂O, and adjust pH to 7.2 with 1 M NaOH.

A2: To buffer A1 add 0.1% V/V Tween 20 and 0.1% V/V pig serum.

B: 0.1 M TRIS-HCL, 0.1% V/V Tween 20 and 0.1% V/V pig serum. To 100 ml UHQ water add 1.211 g TRIS base and adjust pH to 7.2 with conc. HCL, add 0.01 ml Tween 20 and 0.1 g pig serum.

Protocol

1. The DEA was diluted 1:4000 to 2.5 µg/ml in bicarbonate buffer pH 9.6. The wells of a 96-well microtitre plate were coated with 250 ng DEA in 100µl bicarbonate buffer overnight at 4 °C in the dark.
2. Desmosine and isodesmosine standards were dissolved in Buffer B to yield a range of concentrations of 0, 5, 10, 50, 100, 500, 1000 and 2000 ng/ml buffer B.

3. Rabbit anti-desmosine serum was diluted 1:3000 in Buffer B.
4. Standards and samples (100µl) were added to 200µl of rabbit anti-desmosine serum and 50µl buffer B and incubated at 37 oC for 30 minutes, then placed on ice to cool.
5. Plates were washed 6x with 150 µl PBS (phosphate-buffered saline)/ 0.1% Tween 20.
6. Standards and samples (100 µl in duplicate) were added to the wells of the microtitre plate and the plate covered and incubated for 2hr at 4 oC.
7. Plates were washed 6x with 150 µl PBS/ 0.1% Tween 20.
8. Biotinylated swine anti-rabbit (Dako) was diluted 1:1000 in Buffer A2 and 100 µl added to each well and left to incubate for 1hr at room temperature.
9. SAB complexes (Dako) were prepared at 1:100 dilution (1 µl A plus 1 µl B in 100 µl buffer A2) for 30 minutes at room temperature, then diluted to 10 ml in buffer A2 for use.
10. Plates were washed 6x as before and 100 µl SAB complexes added to each well and left for 30 minutes at room temperature.
11. Substrate (5.5 mM o-phenylene-diamine solution in TRIS- citrate buffer pH 6) was prepared; to 50 ml UHQ water add 1.21 g TRIS, 0.6 g citrate, 50 µl 30% H2O2, 0.03 g o-phenylene diamine.
12. Plates were washed 6x and 100 µl of the substrate solution added to each well and colour allowed to develop for 30 minutes at 37 oC.
13. Reactions were stopped by the addition of 100 µl 2 M H2SO4 and the plate read at 490 nm.

Gelatinase/Collagenase Assay

The following assay reagents procured from:

Molecular Probes Europe BV
Poortgebouw
Rijnsburgerweg 10 2333 AA Leiden
The Netherlands

Phone: = +31-71-5233378

www.probes.com

The following assay was developed from these key references:

- Alberts, B et al., Molecular Biology of the cell, 2nd Edition, Garland Publishing, inc. (1989) pp. 802-824.
- Chem Biol 3, 895 (1996)
- J Biol Chem 270, 5872 (1995)
- Lab Invest 78, 687 (1998)
- Anticancer Res 19, 1809 (1999)

Reagents:

- DQ gelatin from pig skin, fluorescein conjugate, (component A) 1 mg substrate lyophilised from 1 ml of phosphate-buffered saline (PBS), dissolved into 1 ml UHQ water to give 1 mg/ml stock pH 7.2.
- 10x Reaction Buffer, (component B) 50 ml of 0.5 M Tris-HCL, 1.5 M NaCl, 50 mM CaCl₂, 2 mM Sodium Azide, pH 7.6.
- Collagenase, type 4 from Clostridium histolyticum, (component C) 1000 U/ml, one unit (U) is defined as the amount of enzyme required to liberate 1 µmole of L-leucine equivalents from collagen in 5 hrs at 37 °C, pH 7.5.

Reagent Preparation

- Component A: Dissolve DQ gelatin stock (1 mg/ml) 1:10 with reaction buffer, giving a solution of 0.1 mg/ml, 100 µl DQ gelatin + 900µl reaction buffer. (N.B. make up component B “reaction buffer” before attempting preparation of component A).
- Component B: Prepare 1x reaction buffer by diluting 1:10 with UHQ water, add 2 ml of (x10) reaction buffer to 18 ml of UHQ water.
- Component C: Using 5 µl stock collagenase (1000 U/ml) dilute 1:1000 in 5 ml of reaction buffer yielding a 1 U/ml collagenase solution.

Be advised, all reagents must be kept on ice whenever possible throughout the preparation process, until fluorescence is being read during reaction.

Protocol

1. Dilute down 1:2 Component C (Collagenase 1 U/ml) with Component B (reaction buffer) by adding 0.5 ml of component C to 0.5 ml of component B yielding a series of decreasing concentrations 1, 0.5, 0.25, 0.125 and 0 U/ml.
2. Pipette 20 µl of Component A (DQ gelatin 0.1 mg/ml) into each assay well of a 96 well microtiter plate
3. Pipette 80 µl of Component B (1x Reaction Buffer) into each assay well of the microtiter plate.
4. Add the 100 µl of samples and varying concentrations of Component C (Collagenase) to individual assay wells containing Components A and B. (N.B. the reaction will have started as soon as component C is added, so keep the microtiter plate on ice and add the standards as efficiently as possible to reduce error)
5. Take preparations off ice and measure increasing fluorescence as a consequence of Fluorescein conjugate liberation; due to gelatine breakdown, measure fluorescence periodically every 10 minutes over the next two hours.

Neutrophil elastase (NE) activity

Assay buffer (0.3M TRIS-HCl, containing 1.5M NaCl, pH 8.0).

0.3M TRIS base - 0.36 g/10ml

1.5M NaCl - 0.87 g/10ml

Add conc HCl to pH 8.0, H₂O to 10ml.

Substrate;

N-methoxysuccinyl-ala-ala-pro-val-p-nitroanilide is made up as a concentrated stock solution in dimethylsulphoxide (DMSO) at 88.9 mM (ie 10 mg/190µl) and stored in 20µl aliquots at -20°C.

On the day of assay, aliquots were diluted to 3.2ml in assay buffer, as required.

NE activity was measured in BAL samples that had been thawed once only.

BALF samples (10µl) were placed in the wells of a 96-well microtitre plate, and pre-incubated for one minute at 37°C. Then 90µl substrate (0.555mM M-methoxysuccinyl-ala-ala-pro-val-p-nitroanilide (Sigma, UK) in assay buffer) was added and the plate incubated over night at 37°C. The colour change was read as an increase in absorbance at 410nm using a microtitre plate reader (Dynatech, UK).

BALF myeloperoxidase assay

1. OPD substrate;

0.09g o-phenylene diamine dissolved in 50 ml of 50mM phosphate-citrate buffer (Sigma), and add 4µl of 30% H₂O₂ to 50 ml substrate solution.

2. Phosphate-citrate buffer

50 mM sodium phosphate Na₂HPO₄; 0.071g in 100 ml water

50mM citric acid; 0.961g in 100 ml water

Adjust the pH of sodium phosphate solution with the citric acid solution to pH 5.0.

3. Assay;

Incubate 100 µl of sample with 100 µl substrate in phosphate-citrate buffer.

Incubate at 37°C until a strong colour develops.

The reaction was stopped by the addition of 50µl 2M Sulphuric acid (H₂SO₄) and the plate read at 490nm.

Results

Urinary desmosine levels increased significantly at 4, 6, 8, and 10 h post sulfur mustard challenge, but there were no significant effects post-air exposure (Fig 1a). Desmosine levels in the terminal bronchoalveolar lavage (BAL) were not significantly different to those in air (Figure 1b).

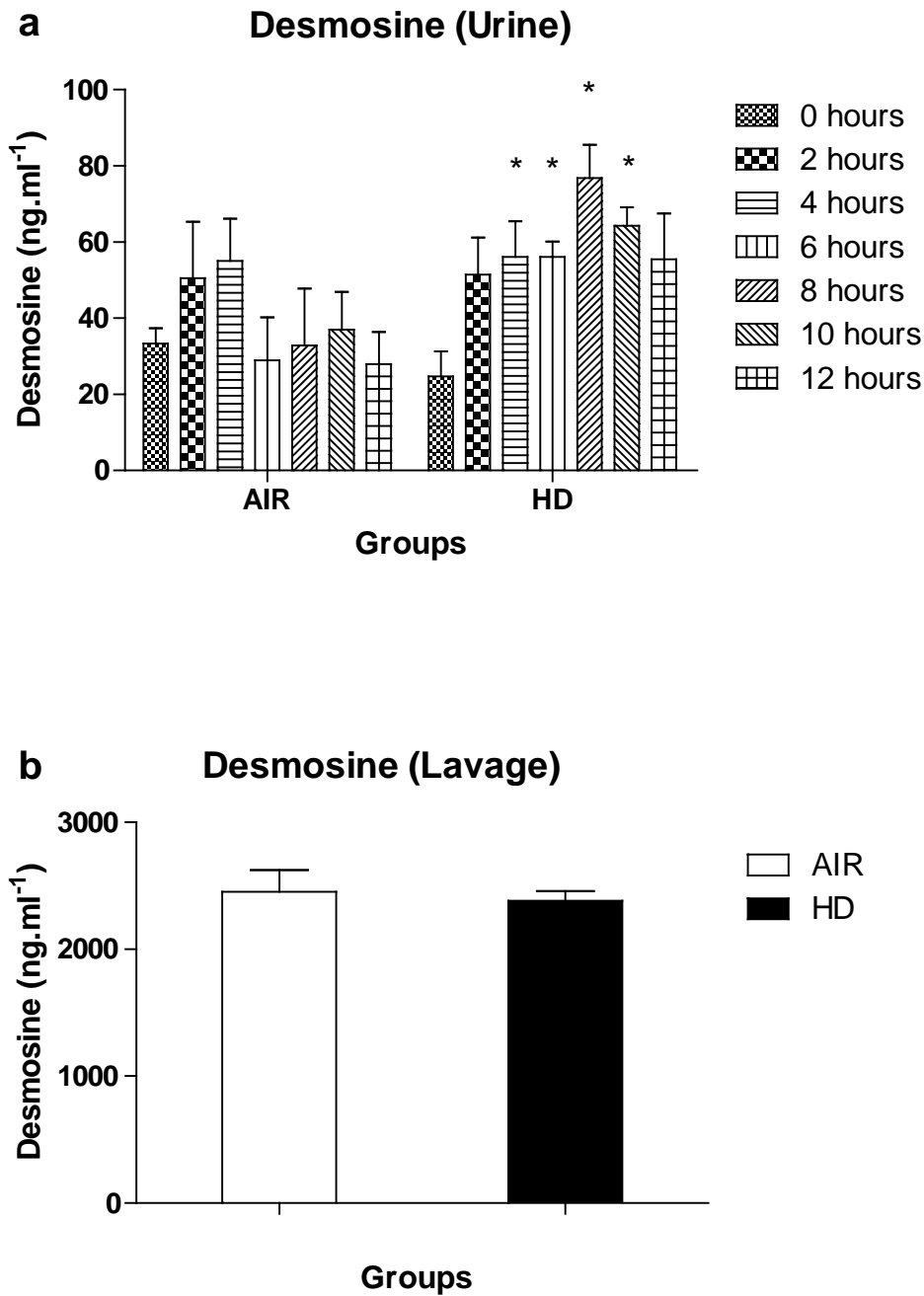


Figure 1; Desmosine concentrations in a) urine or b) bronchoalveolar lavage post exposure to air or HD (n=6 in each group).

However, the BAL desmosine concentrations correlated significantly with BAL neutrophil numbers, both as a percentage of total cells and absolute numbers of neutrophils (Figure 2).

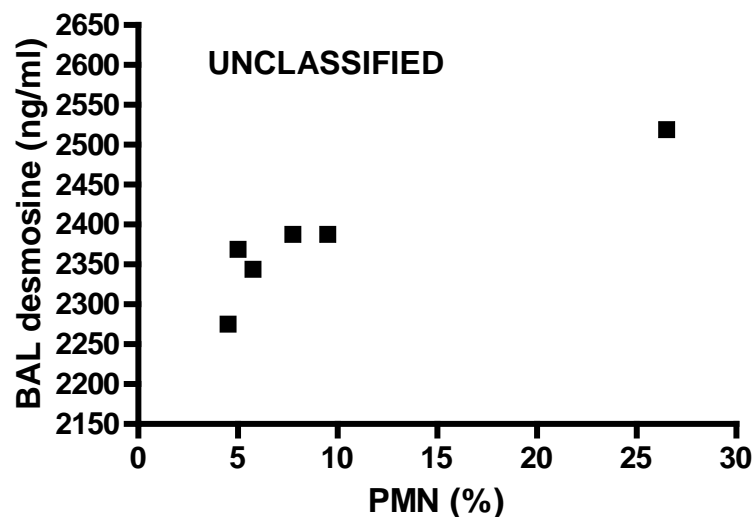


Figure 2; The correlation of BAL desmosine levels with neutrophil % from differential cell counts. ($r^2 = 0.86$, $p=0.167$).

The same correlation was not seen in the air challenged group.

No correlation of urinary desmosines at any time point with BAL biomarkers (neutrophil numbers, IL-8, neutrophil elastase, myeloperoxidase (MPO) or matrix metalloproteinase (MMP)) or plasma MMP activity at any time point could be found in the HD challenged group.

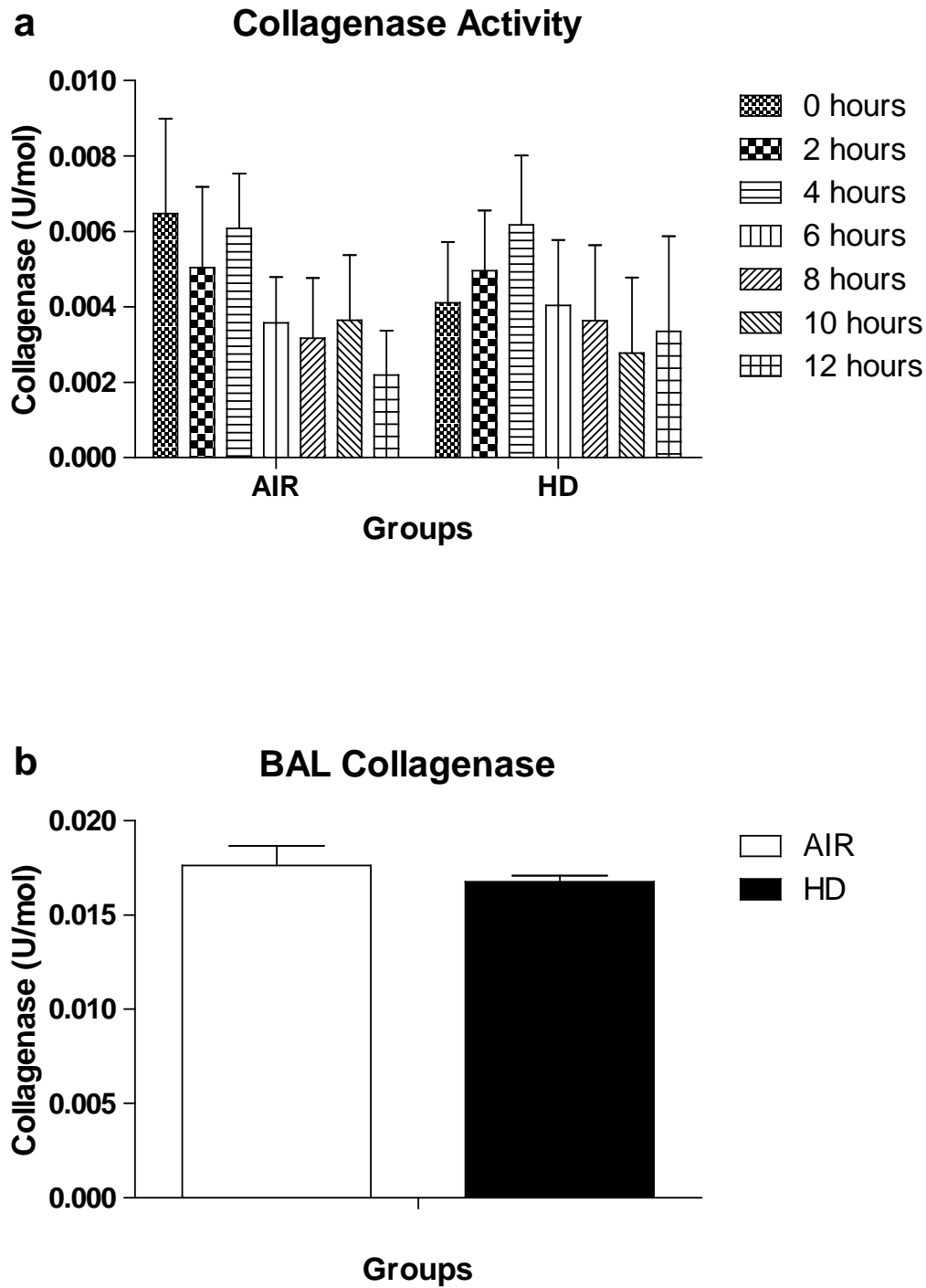


Figure 3; There were no significant differences in MMP activity measured as collagenase activity in a) plasma and b) bronchoalveolar lavage (n=6 in each group).

There were no significant differences in MPO activity (Figure 4) or in elastase activity (Figure 5), although trends towards increase in elastase and MPO activity (markers of neutrophil activation) can be seen in the terminal lavage between groups.

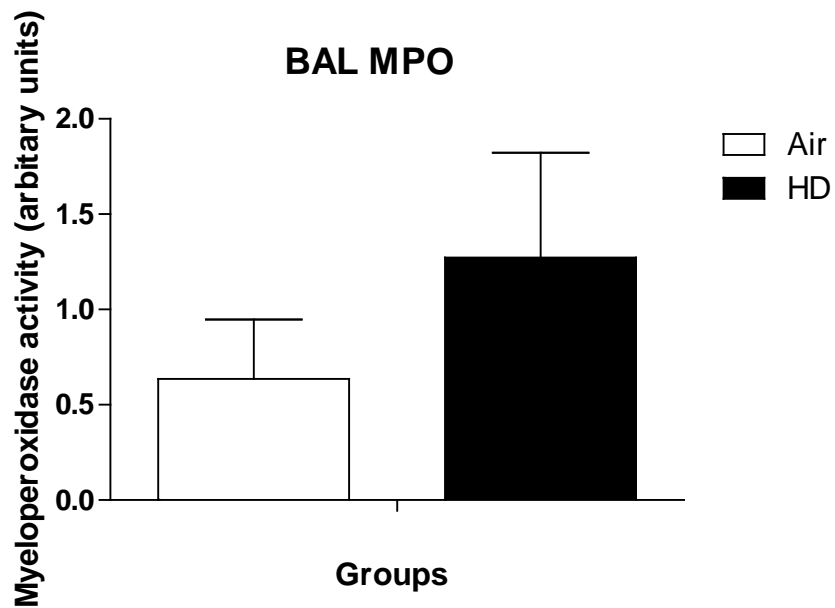


Figure 4; Myeloperoxidase activity in BAL fluid. (n=6 in each group).

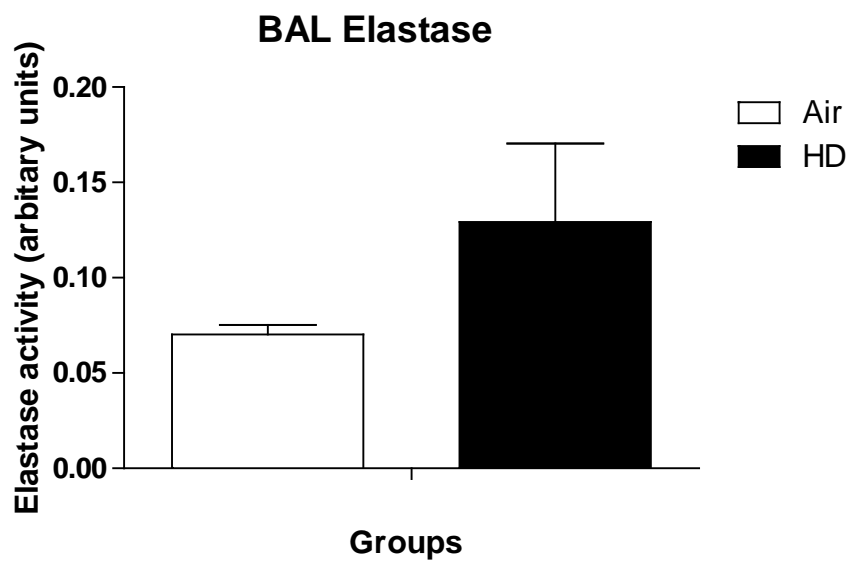


Figure 5; Neutrophil elastase activity in BAL fluid. Using the Mann Whitney U-test, $p=0.065$, (n=6 in each group).

Conclusions

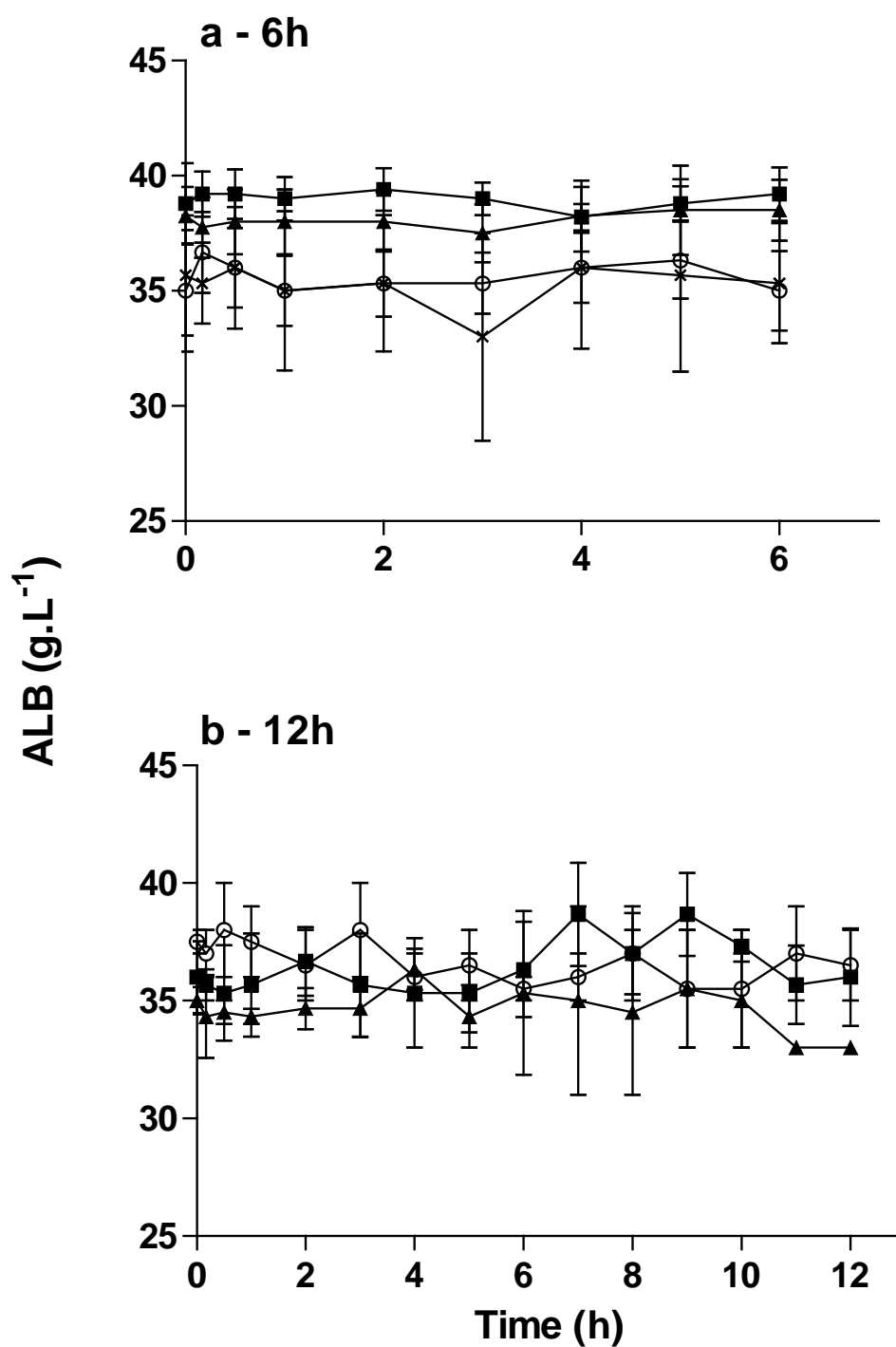
The results provide evidence of neutrophil activation in the lung and elastase-mediated breakdown of the matrix protein elastin, which can be monitored as desmosine in urine.

ANNEX B Clinical Chemistry methods on IL300 analyser

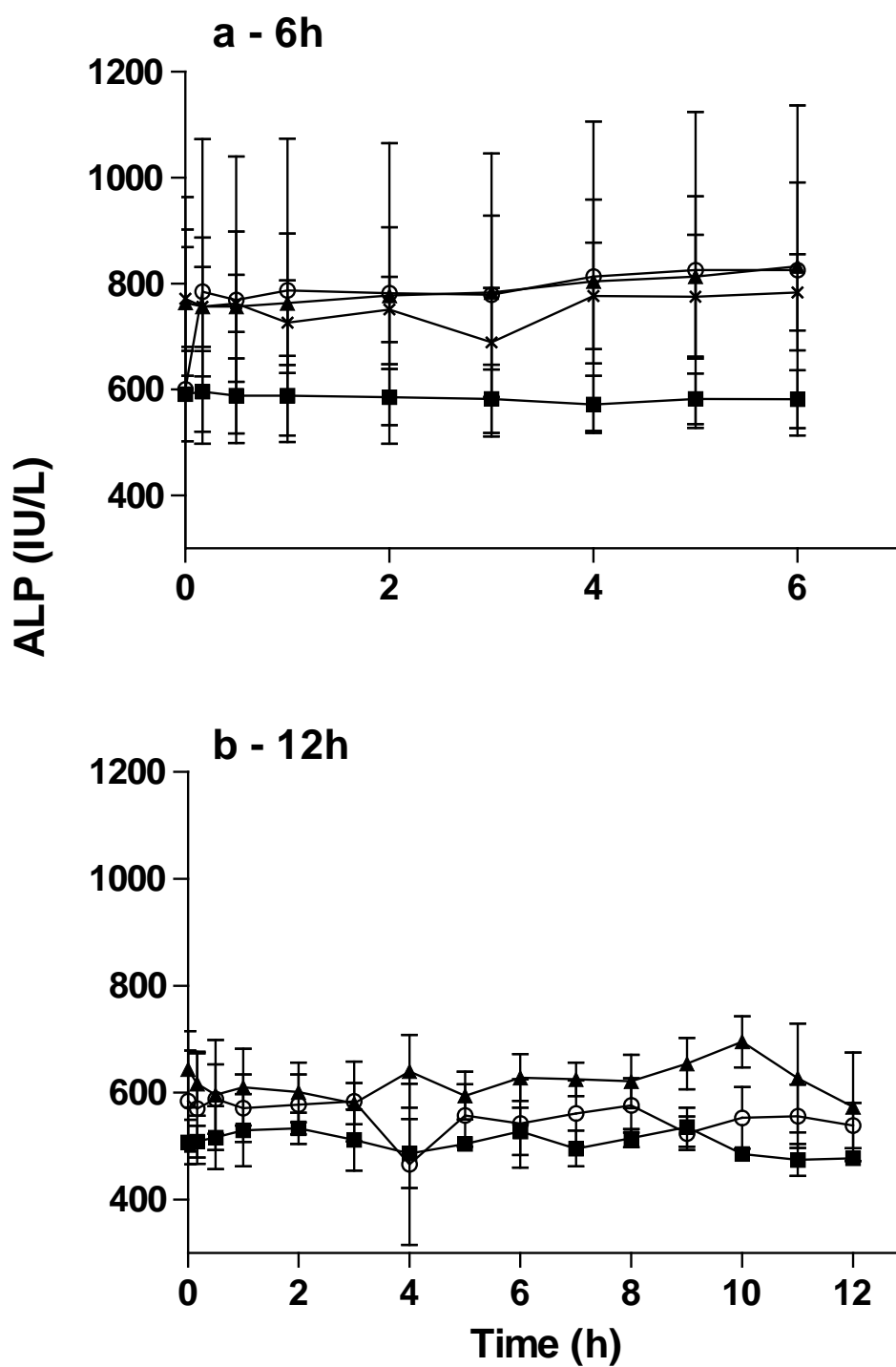
Parameter	Code	Unit	Method	Clinical significance
Albumin	ALB	g/l	Bromocresol Green (BCG)	Decreased levels of albumin may be associated with inflammatory processes or diseases of the liver or kidney.
Alkaline Phosphatase	ALP	U/l	Diethanolamine buffer DEA	Increased in renal disease, liver disease and bone metabolism/bone disease (increased bone turnover).
Alanine Aminotransferase	ALT	U/l	Tris buffer no P5P IFCC, SFBC	Increased in liver cell necrosis of any cause, trauma to skeletal muscle, heat stroke, myocarditis. Slightly more liver- specific than AST.
Aspartate Aminotransferase	AST	U/l	Tris buffer no P5P IFCC, SFBC	Increased in liver cell necrosis, hepatitis or liver injury. Increased in heart or skeletal muscle disease or trauma. Increased in vitro by haemolysis (present in RBC)
Bilirubin, Direct	D Bili	µmol/l	Diazo with Sulphanilic acid	Conjugated Bilirubin (Direct Bili) increased in biliary tree obstruction and some cases of hepatocellular damage.
Bilirubin, Total	T Bili	µmol/l	Diazo with Sulphanilic acid	Increased in hepatocellular damage, biliary tree obstruction, haemolytic diseases.
Calcium (total)	Ca	mmol/l	Cresolphthalein complexone	<i>Total</i> calcium is of limited value during operative procedures due to pH changes and use of resusc fluids/blood products, and changes in albumin concentrations (as most calcium is albumin-bound)
Creatine kinase	CK	U/l	CK-NAC	Increased in skeletal muscle disease and muscle trauma, but also increased in cardiac damage.
Creatinine plasma	Creat. P	µmol/l	Alkaline picrate without deproteinisation	Increased in renal disease.
Creatinine urine	Creat. U		Alkaline picrate without deproteinisation	Decreased in renal disease, hyperthyroidism, anaemia, muscle atrophy.
Gamma Glutamyl Transferase	GGT	U/l	Gamma glut'3-carb'4-nitro	Liver disease indicator (more obstruction than cell damage/death).
Glucose	Gluc	mmol/l	Glucose oxidase	Increased in severe metabolic stress.
Protein, Total	TP	g/l	Biuret reaction, end point	Changes in a variety of liver and kidney diseases as well as metabolic and nutritional disorders, and state of hydration.
Urea	Urea	mmol/l	Urease, kinetic	Increased in renal disease.

NB Clinical significance information is incomplete but pertains to acute illness/operative/recovery scenarios. All parameters may be affected by severe changes of hydration state, blood volume etc as well as actual disease processes.

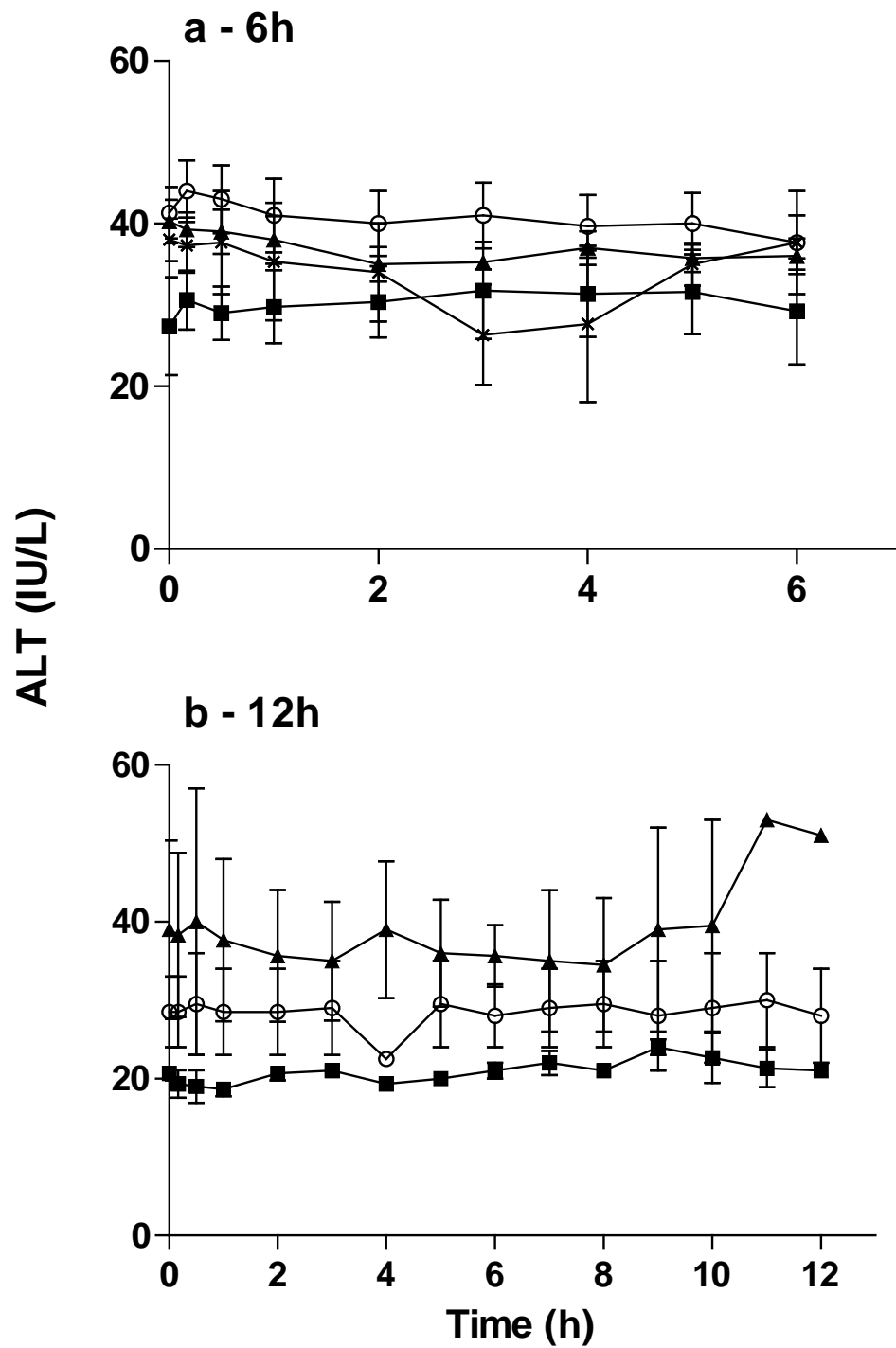
Results



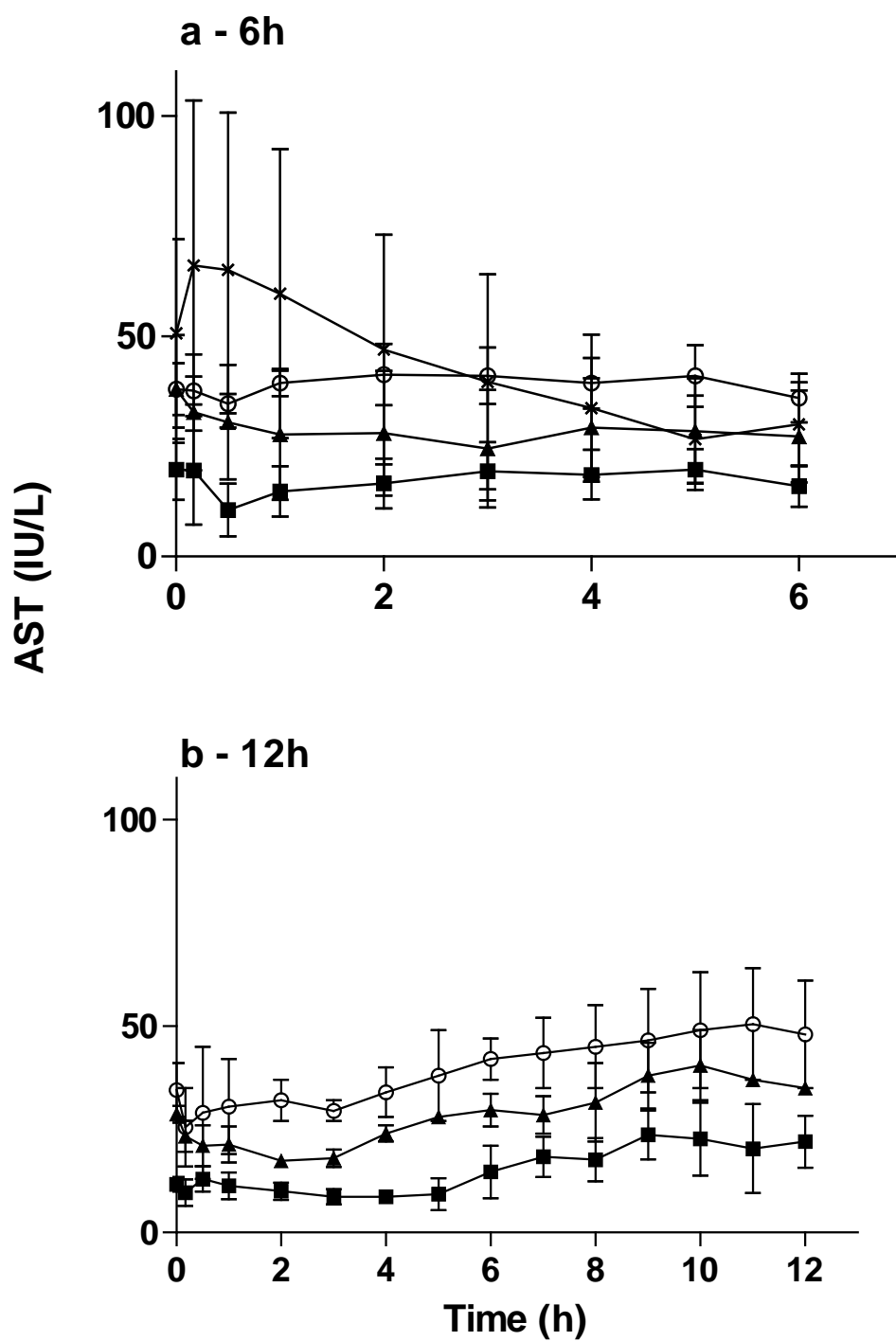
Changes in ALB following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



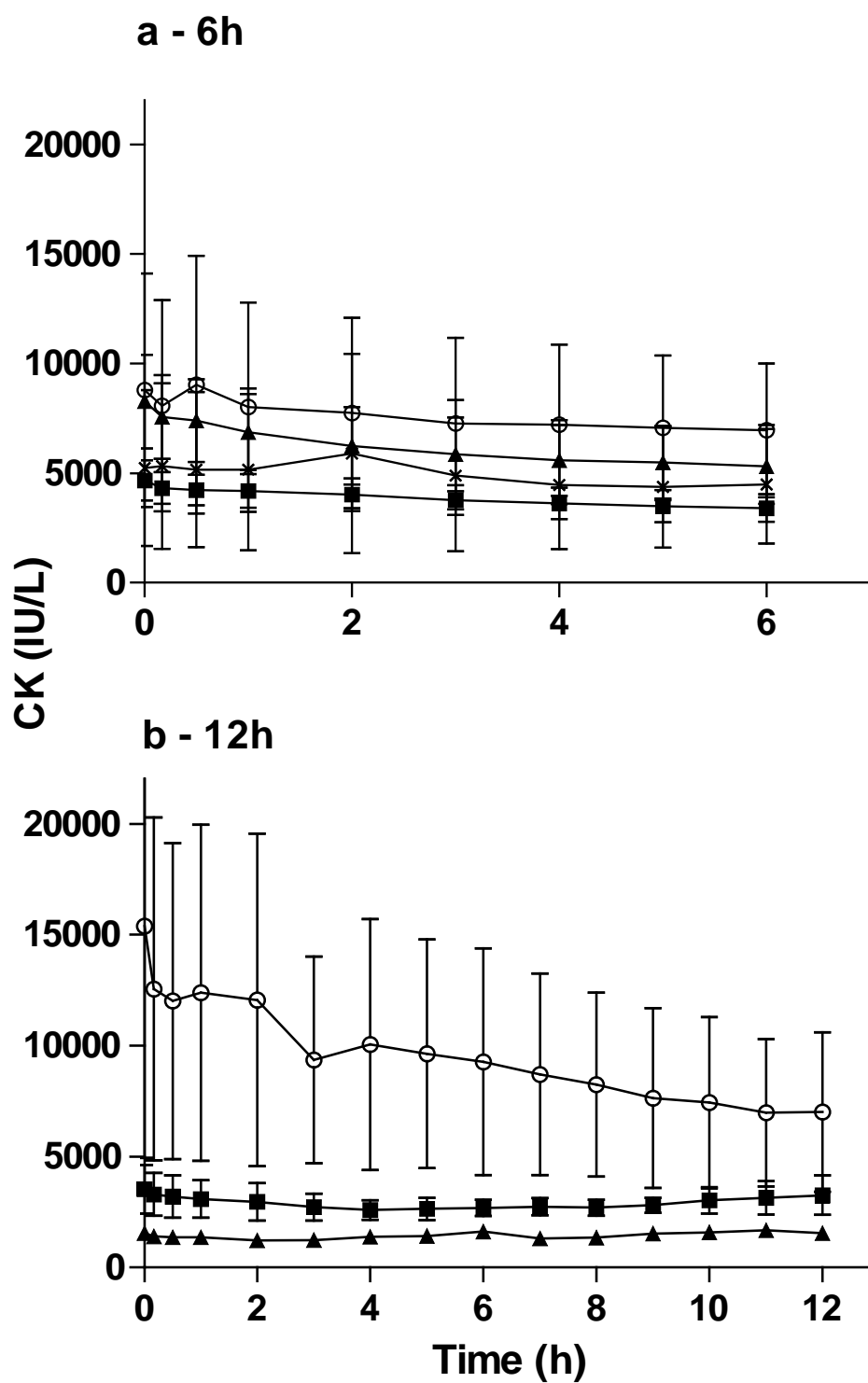
Changes in ALP following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



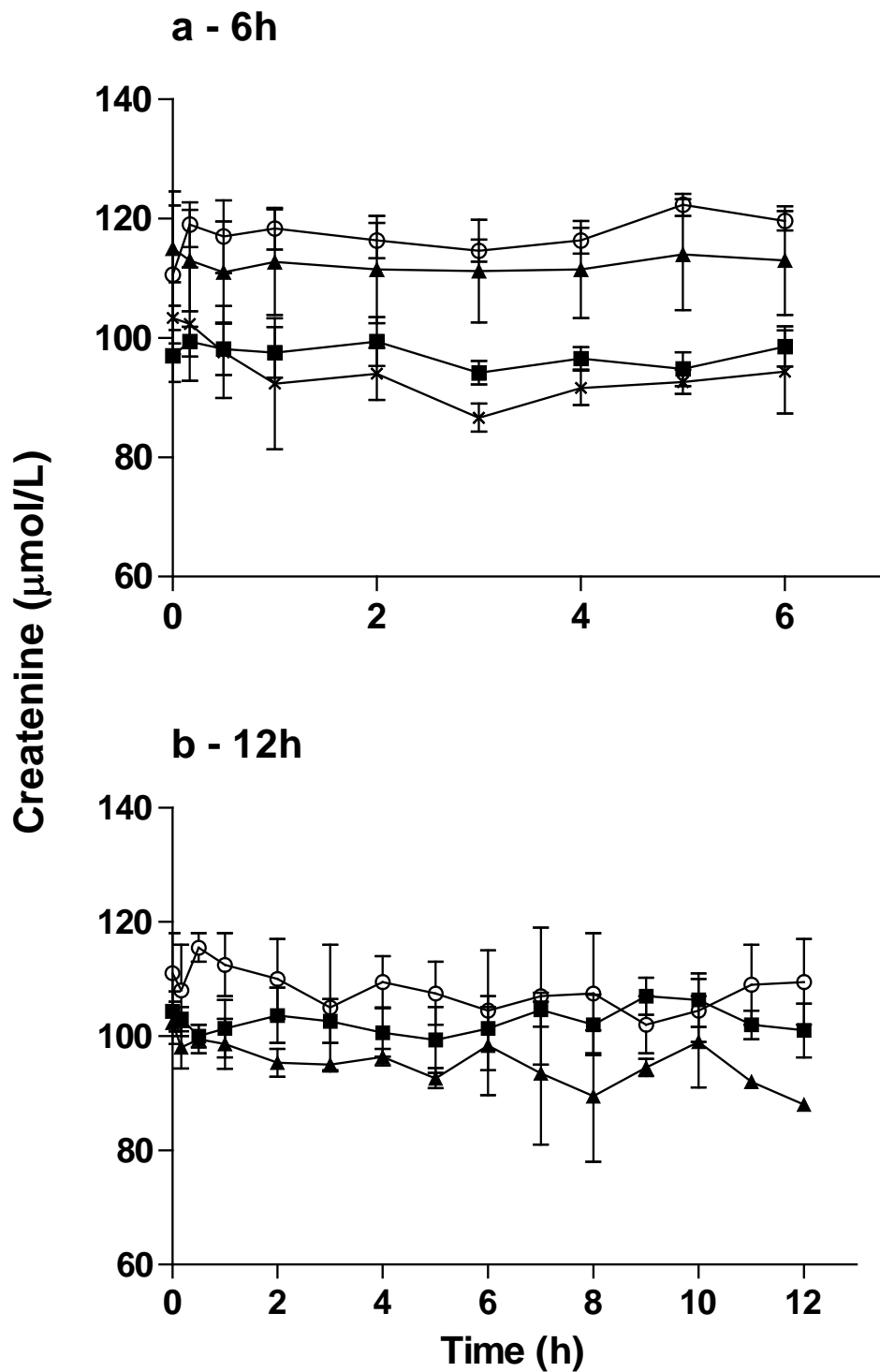
Changes in ALT following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



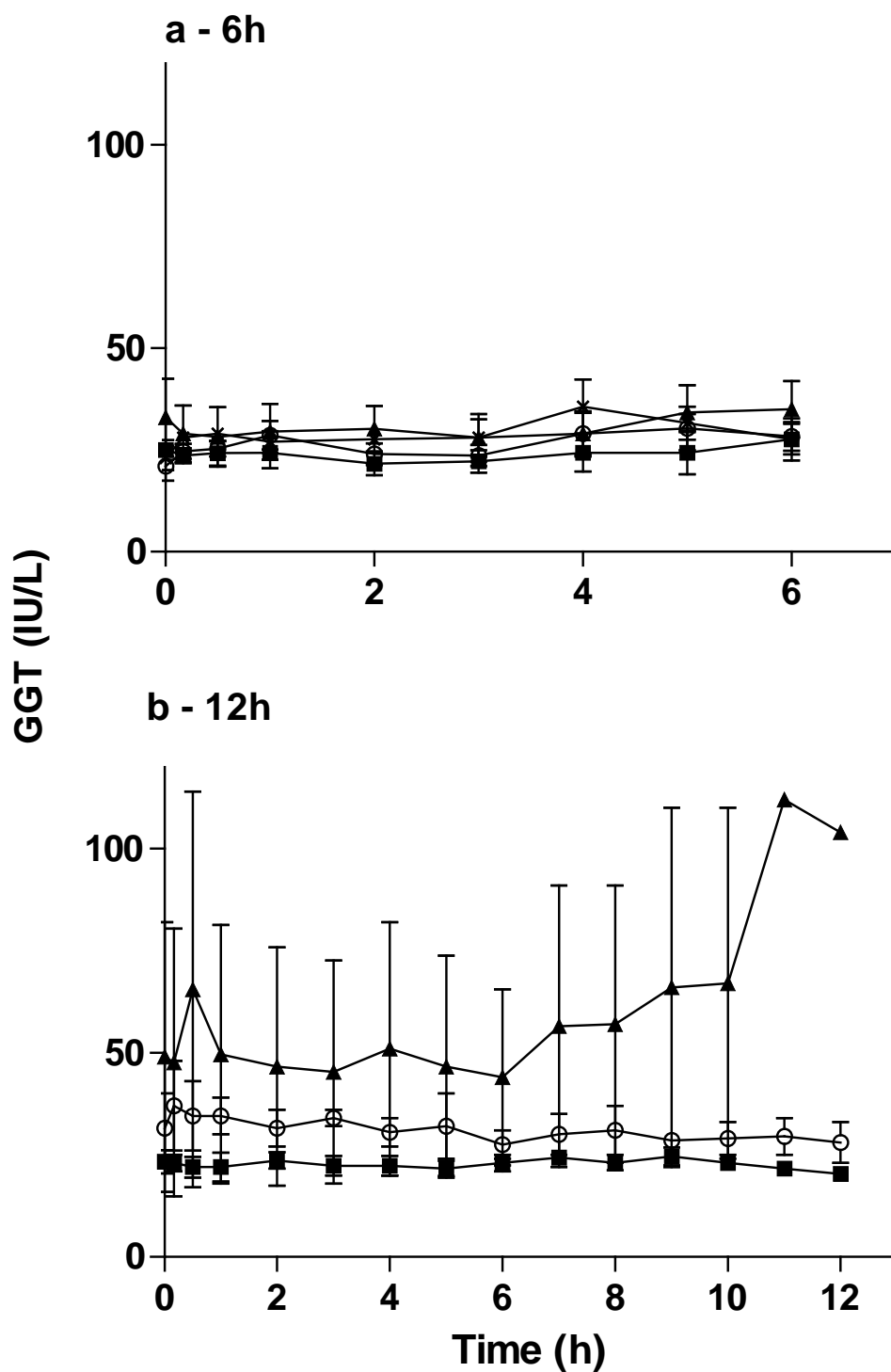
Changes in AST following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (○). Data are expressed as mean \pm SE.



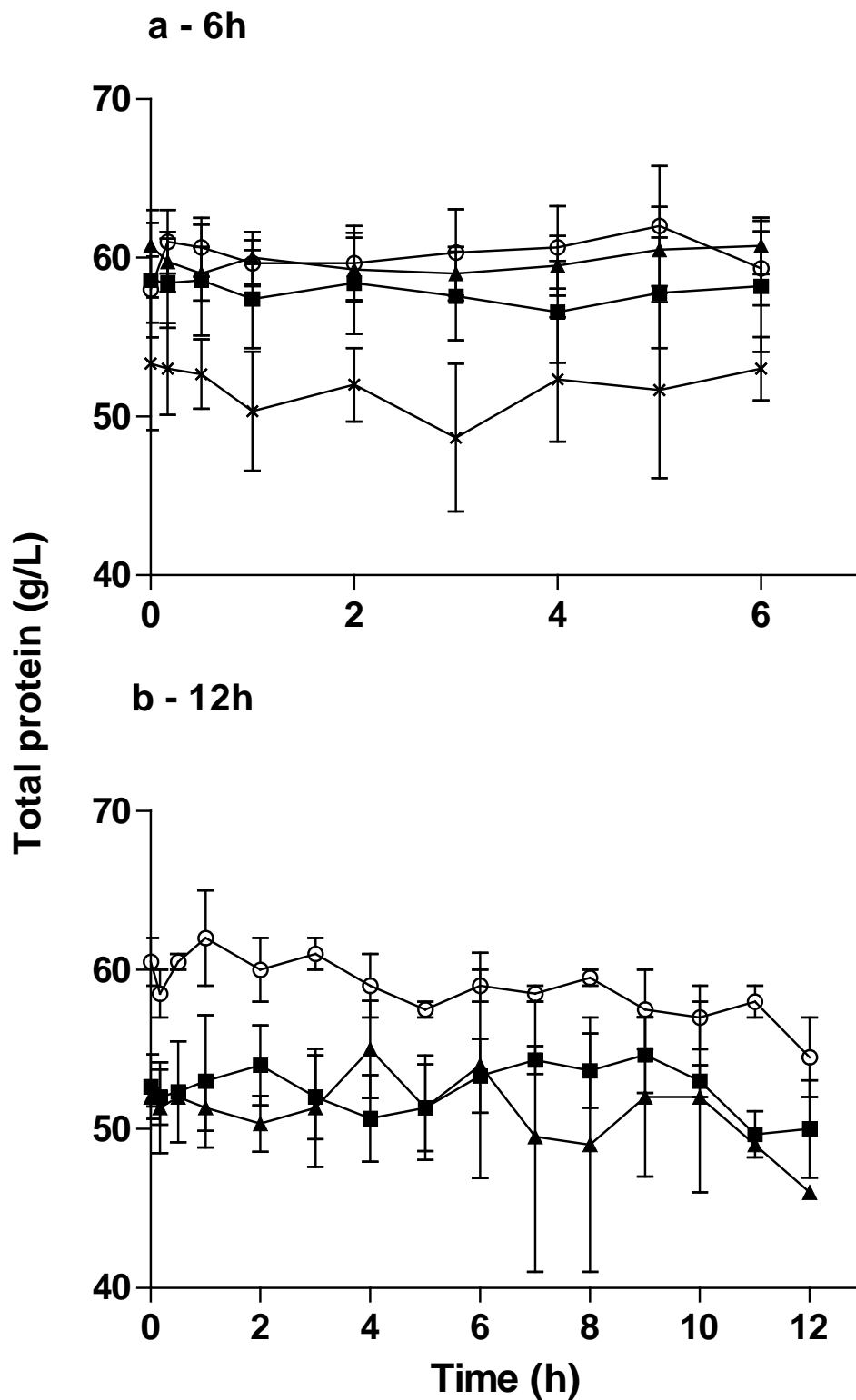
Changes in CK following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



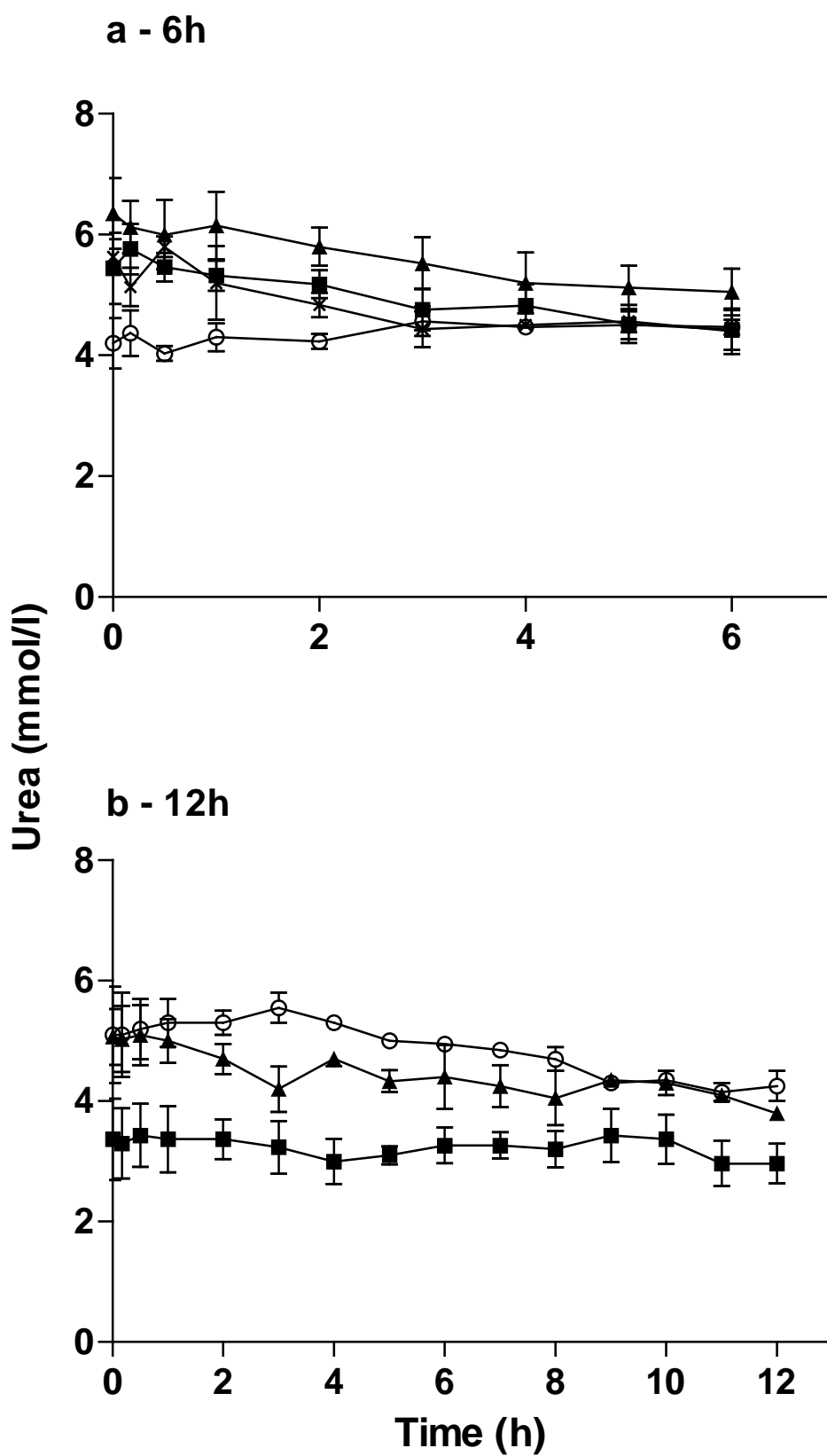
Changes in creatinine following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean ± SE



Changes in GGT following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



Changes in total protein following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



Changes in urea following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.

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ANNEX C Statistical analysis for 6 hour inhaled HD study

Kay Bridgwater

This annex summarises the statistical analysis of data collected to investigate the physiological consequences of exposure to inhaled HD. The study included a control group (n=4) and 3 groups exposed to HD at various concentrations (Low = 60µg.Kg⁻¹, n=5; Medium = 100 µg.Kg⁻¹ n=5; High = 150 µg.Kg⁻¹ n=5). Various measurements were taken over time and the objective of the statistical analysis is to make a comparison between the Air control group and the groups exposed to HD.

Area Under the Curve

The data, compiled into binned time periods 0-3hrs and 3-6hrs, was checked for normality using an Anderson-Darling test. The majority of the data was not normal however some could be transformed using a log transformation to result in a normal data set.

Normally distributed data was analysed via t-test and data not normally distributed was analysed using a non-parametric Mann-Whitney U test. Significant results are highlighted in table 1.

Table 1

Parameter	Comparison	P value
Arterial base excess	Air (0-3h) Vs Low (0-3h)	0.616
	Air (0-3h) Vs Medium (0-3h)	0.873
	Air (0-3h) Vs Medium (0-3h)	0.461
	Air (3-6h) Vs Low (3-6h)	0.78
	Air (3-6h) Vs Medium (3-6h)	0.576
	Air (3-6h) Vs Medium (3-6h)	0.021
Arterial bicarbonate	Air (0-3h) Vs Low (0-3h)	0.87
	Air (0-3h) Vs Medium (0-3h)	0.745
	Air (0-3h) Vs Medium (0-3h)	0.106
	Air (3-6h) Vs Low (3-6h)	0.662
	Air (3-6h) Vs Medium (3-6h)	0.927
	Air (3-6h) Vs Medium (3-6h)	0.0373
Arterial oxygen tension	Air (0-3h) Vs Low (0-3h)	0.604
	Air (0-3h) Vs Medium (0-3h)	0.455
	Air (0-3h) Vs Medium (0-3h)	0.333
	Air (3-6h) Vs Low (3-6h)	0.416
	Air (3-6h) Vs Medium (3-6h)	0.017
	Air (3-6h) Vs Medium (3-6h)	0.015

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Arterial potassium	Air (0-3h) Vs Low (0-3h)	0.049
	Air (0-3h) Vs Medium (0-3h)	0.145
	Air (0-3h) Vs Medium (0-3h)	0.185
	Air (3-6h) Vs Low (3-6h)	0.018
	Air (3-6h) Vs Medium (3-6h)	0.0662
	Air (3-6h) Vs Medium (3-6h)	0.133
Arterial standard bicarbonate	Air (0-3h) Vs Low (0-3h)	0.028
	Air (0-3h) Vs Medium (0-3h)	0.016
	Air (0-3h) Vs Medium (0-3h)	0.006
	Air (3-6h) Vs Low (3-6h)	0.069
	Air (3-6h) Vs Medium (3-6h)	0.0662
	Air (3-6h) Vs Medium (3-6h)	0.076
Oxyhemoglobin	Air (0-3h) Vs Low (0-3h)	0.715
	Air (0-3h) Vs Medium (0-3h)	0.252
	Air (0-3h) Vs Medium (0-3h)	0.39
	Air (3-6h) Vs Low (3-6h)	0.363
	Air (3-6h) Vs Medium (3-6h)	0.03
	Air (3-6h) Vs Medium (3-6h)	0.022
Reduced hemoglobin	Air (0-3h) Vs Low (0-3h)	0.887
	Air (0-3h) Vs Medium (0-3h)	0.201
	Air (0-3h) Vs Medium (0-3h)	0.631
	Air (3-6h) Vs Low (3-6h)	0.295
	Air (3-6h) Vs Medium (3-6h)	0.023
	Air (3-6h) Vs Medium (3-6h)	0.033

Summary Measures

The minimum and maximum of each binned time period was taken to use as a measure for further analysis. A random sample of all the available parameters were taken to see if analyzing this data in a different way would identify any additional results and back up the original t-tests.

General Linear Model

Data that was found to be normally distributed either originally or after a log transformation was put into a General Linear Model and each treatment for each time period, compared to the air control for the same time period.

A non-parametric Kruskal Wallis test was carried out on the data sets which were not normally distributed, with any significant differences being further investigated using Mann –Whitney U tests between the controls and treatments.

There were few significant differences in the sample of parameters tested and the ones that were found (as highlighted in table 2 below) had already been picked up by the original t-tests, therefore it was decided not to continue with this analysis.

Table 2

Parameter	Comparison	P value
Heart rate	Log Min 0-3hrs, Control vs High Dose	0.9856
	Log Min 0-3hrs, Control vs. Medium Dose	0.8462
	Log Min 0-3hrs, Control vs. Low Dose	0.9857
	Log Max 0-3hrs, Control vs. High Dose	0.4983
	Log Max 0-3hrs, Control vs. Medium Dose	0.6391
	Log Max 0-3hrs, Control vs. Low Dose	0.8801
	Log Min 3-6hrs, Control vs. High Dose	0.3544
	Log Min 3-6hrs, Control vs. Medium Dose	0.9906
	Log Min 3-6hrs, Control vs. Low Dose	0.8288
	Log Max 3-6hrs, Control vs. High Dose	0.1095
	Log Max 3-6hrs, Control vs. Medium Dose	0.7123
	Log Max 3-6hrs, Control vs. Low Dose	0.0756
Shunt Fraction	Log Min 0-3hrs, Control vs. High Dose	0.8051
	Log Min 0-3hrs, Control vs. Medium Dose	0.8093
	Log Min 0-3hrs, Control vs. Low Dose	0.2873
	Log Max 0-3hrs, Control vs. High Dose	0.9975
	Log Max 0-3hrs, Control vs. Medium Dose	0.9883
	Log Max 0-3hrs, Control vs. Low Dose	0.8118
	Log Min 3-6hrs, Control vs. High Dose	0.1324
	Log Min 3-6hrs, Control vs. Medium Dose	0.2379
	Log Min 3-6hrs, Control vs. Low Dose	0.0062
	Log Max 3-6hrs, Control vs. High Dose	0.0016
	Log Max 3-6hrs, Control vs. Medium Dose	0.7467
	Log Max 3-6hrs, Control vs. Low Dose	0.0023

Kruskal Wallis

Parameter	Comparison	P value
Hemoglobin	Min 0-3hrs vs. all doses	0.286
	Max 0-3hrs vs. all doses	0.56
	Min 3-6hrs vs. all doses	0.942
	Max 3-6hrs vs. all doses	0.137
RBC	Min 0-3hrs vs. all doses	0.245
	Max 0-3hrs vs. all doses	0.552
	Min 3-6hrs vs. all doses	0.629
	Max 3-6hrs vs. all doses	0.112
Picco	Min 0-3hrs vs. all doses	0.05
	Max 0-3hrs vs. all doses	0.212
	Min 3-6hrs vs. all doses	0.172

	Max 3-6hrs vs. all doses	0.052
Arterial Potassium	Min 0-3hrs vs. all doses	0.838
	Max 0-3hrs vs. all doses	0.822
	Min 3-6hrs vs. all doses	0.213
	Max 3-6hrs vs. all doses	0.925
Mixed Venous O ₂	Min 0-3hrs vs. all doses	0.641
	Max 0-3hrs vs. all doses	0.947
	Min 3-6hrs vs. all doses	0.05
	Max 3-6hrs vs. all doses	0.705
Arterial O ₂ Content	Min 0-3hrs vs. all doses	0.584
	Max 0-3hrs vs. all doses	0.896
	Min 3-6hrs vs. all doses	0.011
	Max 3-6hrs vs. all doses	0.789
Alveolar O ₂ Tension	Min 0-3hrs vs. all doses	0.537
	Max 0-3hrs vs. all doses	0.651
	Min 3-6hrs vs. all doses	0.369
	Max 3-6hrs vs. all doses	0.726

Principal Component Analysis

It was then decided to use Principal Component Analysis to further analyse the whole data set to identify trends and patterns within the dataset. PCA is the simplest of the Eigen value based statistical techniques. It identifies combinations of variables in large data sets that explain the largest amount of variation in the multivariate dataset. The idea is to create new variables to explain as much of the information in the dataset as possible and identify specific patterns of groups contained within the large dataset. The new variables generated are referred to as principal components and these are compared against each other, and each principal component represents different pieces of information. Thus each principal component slices through the dataset in order to determine patterns within the data.

The first 2 components only were used as they were shown to be the most useful. The first two components calculated explained the majority of the variance within the dataset and were therefore of most interest.

The parameters contributing to the component were derived and then analysed to determine the significance of effect. This is to validate the PCA model and to demonstrate which parameters are responsible for effects on output or contributing to a separation in treatment groups.

The appropriate statistical test was carried out depending on whether the data was normal or not – either a general liner model (ANOVA) or a Kruskal Wallis. The following table lists the parameters and dose level showing significant differences.

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Parameter and Principle Component	Comparison	P value
RBC PC2	Control vs. High	0.0222
	Control vs. Medium	0.007
Hemoglobin PC2	Control vs. High	0.0083
	Control vs. Medium	0.0025
Arterial O ₂ Content PC2	Control vs. High	0.0111
Arterial pH PC2	Control vs. High	0.0295
Venous O ₂ Sat PC2	Control vs. High	0.0058
Hematocrit PC2	Control vs. High	0.0179
	Control vs. Medium	0.0106
Arterial O ₂ Tension mmHg PC2	Control vs. High	0.0256
Arterial O ₂ Tension PC2	Control vs. High	0.0045

ANNEX D Statistical analysis for 12 hour inhaled HD study

Ralph Mansson

Introduction

This annex summarises a statistical analysis of data collected to investigate the physiological consequences of exposure to inhaled HD. The study included an air exposed control group (n=6) and a group exposed to 100µg.kg-1 HD (n=7) and various physiological measurements were taken over time. The objective of the statistical analysis is to make a comparison between the Air control group and the group exposed to HD.

1 Methodology

To test for differences in various metrics between the control group and the exposed group (HD).

Equal Variances for Groups

Bartlett's test was used to test whether the variability of the data is consistent between the air controls and HD exposure group as well as whether it is consistent over time.

Time Point Comparisons

The data was investigated at points in time to test for differences between the air controls and HD exposure group.

Normal probability plots were used to decide whether the assumption of a Normal distribution and hence a parametric test was appropriate for the data.

A two-sample t-test was used to compare the average data values for the two groups.

1.1 Arterial Blood Oxygenation (PaO₂)

1.2 Arterial Base Excess

1.3 Continuous Cardiac Output (PICCO)

1.4 Heart Rate

1.5 Shunt Fraction

1.6 Respiratory Rate

1.7 Lavage Differential WBC Count

1.8 Lavage Protein Concentration

The air controls and HD exposure group were compared using a two-sample Wilcoxon test.

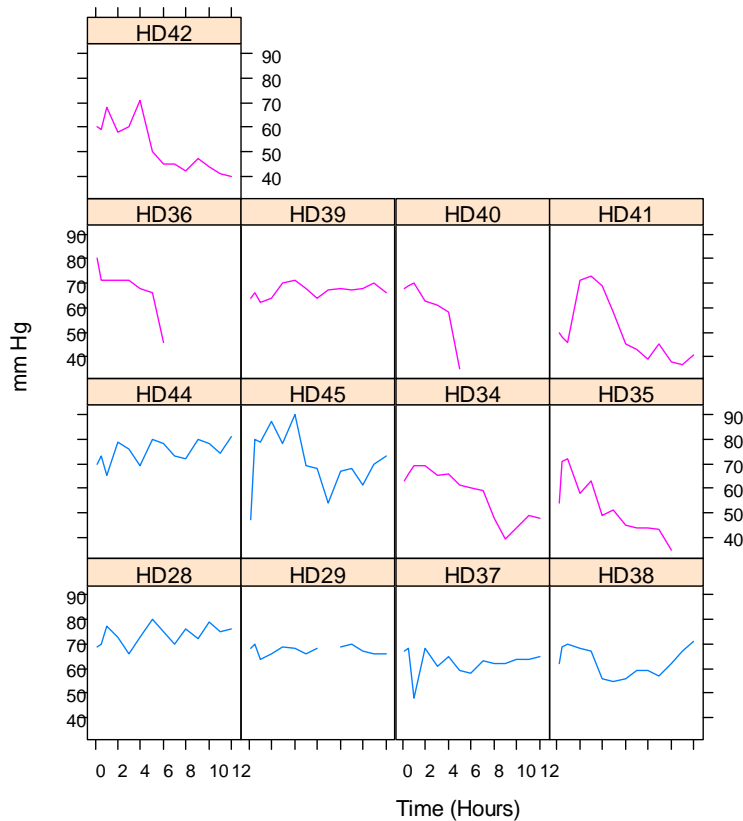
- 1.9 Lung Wet Weight : Body Weight Ratio
- 1.10 Lung Wet Weight : Dry Weight Ratio
- 1.11 Extra Vascular Lung Water Content
- 1.12 Arterial Blood Sodium Concentration
- 1.13 Arterial Blood Lactate Concentration
- 1.14 Arterial Blood Bicarbonate Concentration
- 1.15 Arterial Blood Oxyhemoglobin
- 1.16 Arterial Blood Hematocrit

2 Results

2.1 Arterial Blood Oxygenation (PaO₂)

A graph of the data shown for individual animals indicates that the PaO₂ stays consistent for the air control group (blue lines) and there is a general trend of a decrease for the HD exposure group (pink lines):

Arterial Blood Oxygenation by Anima



Bartlett's test indicated that the variances for the groups and time periods were not consistent.

Normal probability plots for the data in the two groups suggested that this approximation was generally a reasonable assumption at the time points 3, 6, 9 and 12 hours.

Air control versus HD exposure group comparisons:

At 3 hours there is no significant difference between the two groups (p-value = 0.33).

At 6 hours there is a significant difference between the two groups (p-value = 0.009).

At 9 hours there is a significant difference between the two groups (p-value = 0.011).

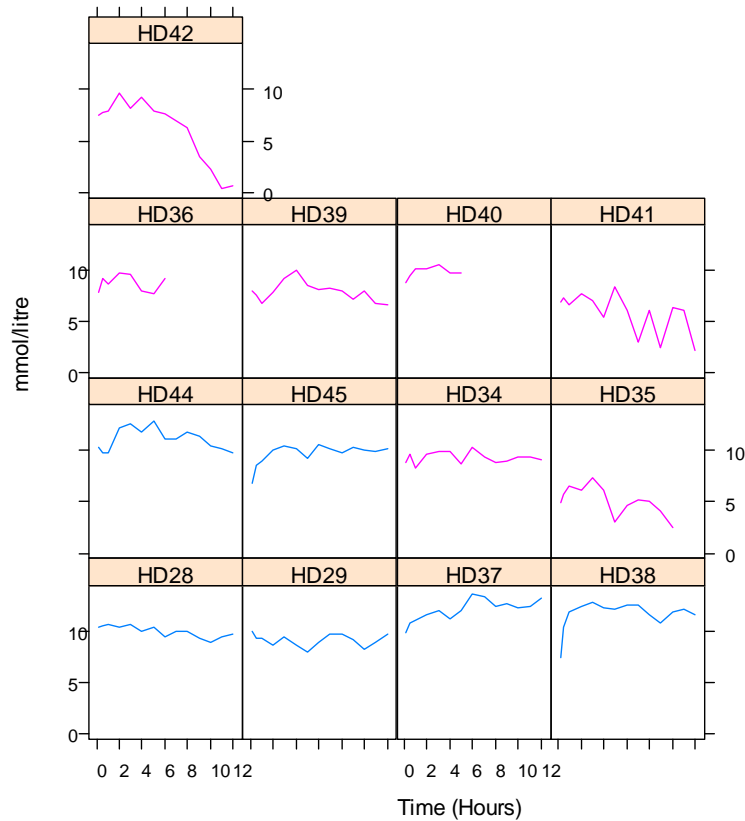
At 12 hours there is a significant difference between the two groups (p-value = 0.023).

2.2 Arterial Base Excess

A graph of Arterial Base Excess against time for each of the animals shows consistency between the animals in the Air control group (blue lines). For the HD exposure group (pink lines) some of the animals died before the end of the 12 hour period and they died

before there was sufficient time for the treatment to have an impact on the Arterial Base Excess:

Arterial Base Excess by Animal



Bartlett's test indicated that the variances for the groups and time periods were not consistent.

Normal probability plots for the data in the two groups suggested that this approximation was a reasonable assumption at the time points 3, 6, 9 and 12 hours.

Air control versus HD exposure group comparisons:

At 3 hours there is a significant difference between the two groups (p-value = 0.0066).

At 6 hours there is a significant difference between the two groups (p-value = 0.013).

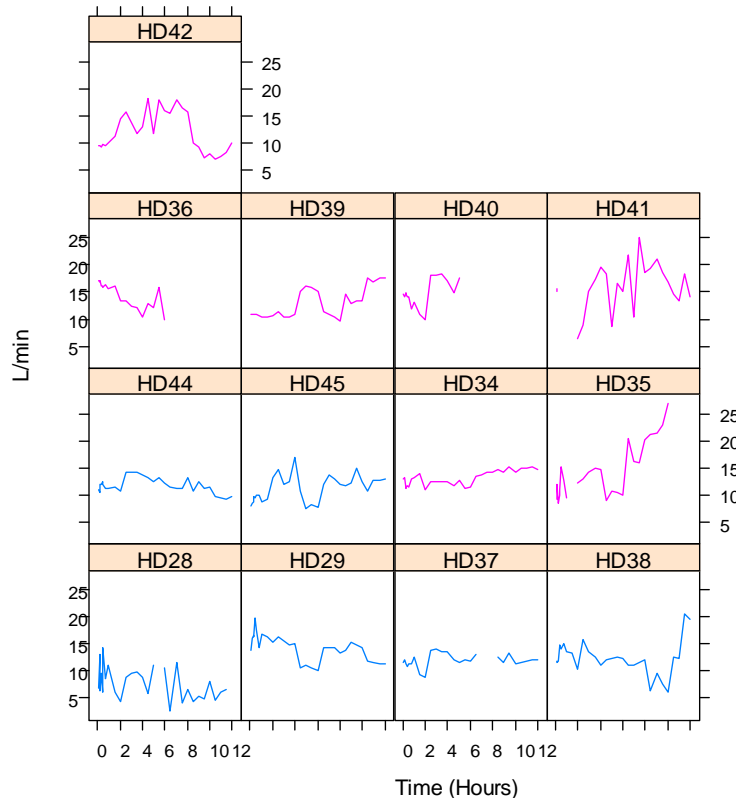
At 9 hours there is a significant difference between the two groups (p-value = 0.0084).

At 12 hours there is a significant difference between the two groups (p-value = 0.049).

2.3 Continuous Cardiac Output (PiCCO)

A graph of Continuous Cardiac Output against time for each of the animals shows consistency across time for the air control group (blue lines) and for the HD exposure group (pink lines) there appears to be greater variation but no consistent pattern of increasing PICCO against time. It should be noted as with the previous measures some of the animals in the HD exposure group may have died before an effect could have been observed.

Continuous Cardiac Output (PICCO)



Bartlett's test indicated that the variances for the groups and time periods were not consistent.

Normal probability plots for the data in the two groups suggested that this approximation was a reasonable assumption at the time points 3, 6, 9 and 12 hours.

Air control versus HD exposure group comparisons:

At 3 hours there is no significant difference between the two groups (p-value = 0.63).

At 6 hours there is no significant difference between the two groups (p-value = 0.15).

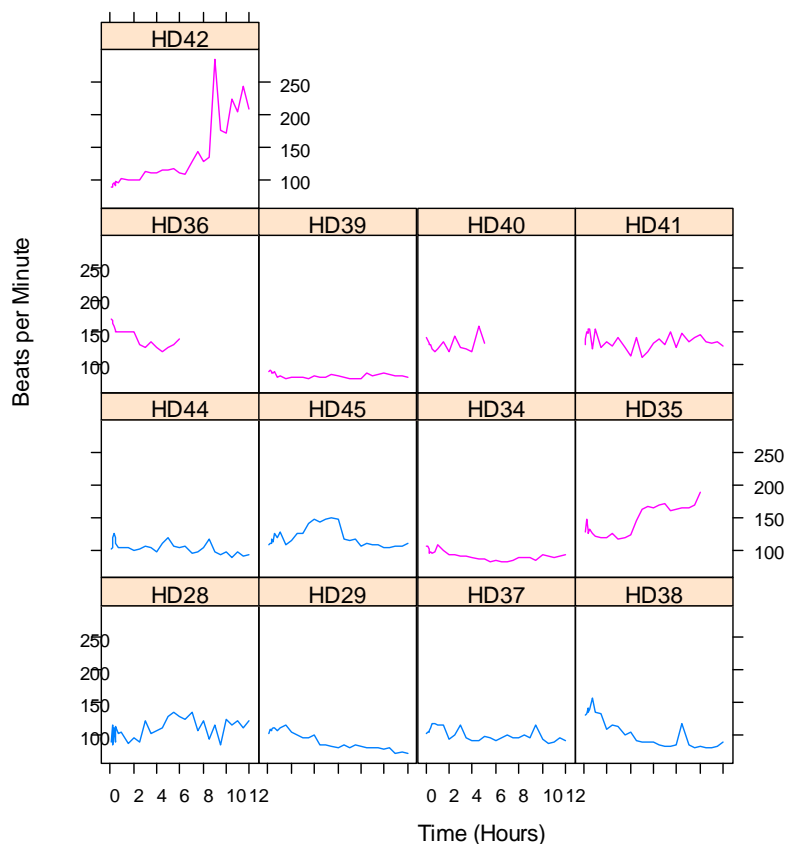
At 9 hours there is no significant difference between the two groups (p-value = 0.14).

At 12 hours there is no significant difference between the two groups (p-value = 0.69).

2.4 Heart Rate

A graph of Heart Rate against time for each of the animals shows consistency across time for the Air control group (blue lines) and for the HD exposure group (pink lines) there appears to be no consistent pattern of increasing Heart Rate against time. It should be noted as with the previous measures some of the animals in the HD exposure group may have died before an effect could have been observed.

Heart Rate by Animal



Bartlett's test indicated that the variances for the groups and time periods were not consistent.

Normal probability plots for the data in the two groups suggested that this approximation was a reasonable assumption at the time points 3, 6, 9 and 12 hours.

Air control versus HD exposure group comparisons:

At 3 hours there is no significant difference between the two groups (p-value = 0.95).

At 6 hours there is no significant difference between the two groups (p-value = 0.48).

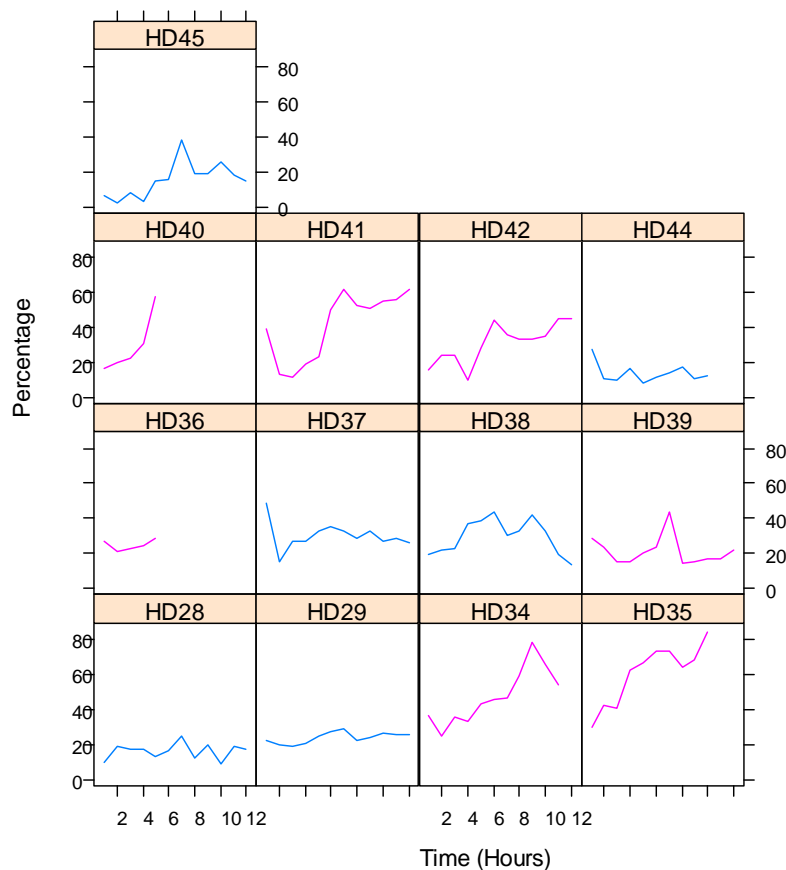
At 9 hours there is no significant difference between the two groups (p-value = 0.22).

At 12 hours there is no significant difference between the two groups (p-value = 0.35).

2.5 Shunt Fraction

A graph of Shunt Fraction against time for each of the animals indicates that the Shunt Fraction stays consistent for the Air control group (blue lines) and there is a general trend of an increase for the HD exposure group (pink lines):

Shunt Fraction by Animal



Barlett's test indicated that the variability was different for the two groups but consistent at specific time points (3, 6, 9 and 12).

Normal probability plots for the data in the two groups suggested that this approximation was broadly a reasonable assumption at the time points 3, 6, 9 and 12 hours.

Air control versus HD exposure group comparisons:

At 3 hours there is no significant difference between the two groups (p-value = 0.16).

At 6 hours there is a significant difference between the two groups (p-value = 0.047).

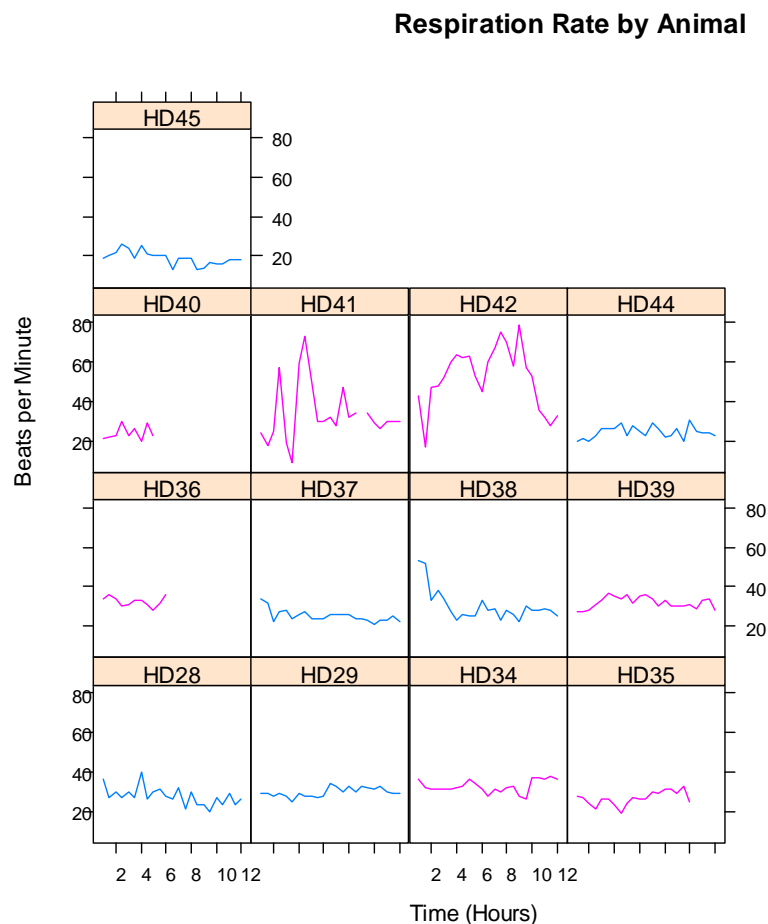
At 9 hours there is no significant difference between the two groups (p-value = 0.10).

At 12 hours there is no significant difference between the two groups (p-value = 0.14).

These results are unexpected as not all of the HD group survive beyond 6 hours. It should be noted that the comparison is between those that do survive so there are fewer data points for the comparison.

2.6 Respiration Rate

A graph of Respiration Rate against time for each of the animals indicates that the Respiration Rate stays consistent for the Air control group (blue lines) and there is no general trend for the HD exposure group (pink lines) with two animals showing a large amount of variability over time:



Bartlett's test shows a mixed picture over time where the variability for the two groups is different at 3 and 9 hours but consistent at 6 and 12 hours.

Normal probability plots for the data in the two groups suggested that this approximation was broadly a reasonable assumption at the time points 3, 6, 9 and 12 hours.

Air control versus HD exposure group comparisons:

3 hours there is no significant difference between the two groups (p-value = 0.59).

At 6 hours there is a significant difference between the two groups (p-value = 0.046).

At 9 hours there is no significant difference between the two groups (p-value = 0.25).

At 12 hours there is a significant difference between the two groups (p-value = 0.012)

2.7 Lavage Differential WBC Count

Bartlett's test shows the variability for the two groups is consistent for Lymphocytes, Monocytes and Eosinophils but is different for Neutrophils and Alveolar Macrophages.

Normal probability plots for the data in the two groups suggested that this approximation was a reasonable assumption for the five cell types.

Cell Type:

Neutrophils – no significant difference between the two groups (p-value = 0.081).

Lymphocytes – no significant difference between the two groups (p-value = 0.75).

Alveolar Macrophages – no significant difference between the two groups (p-value = 0.16).

Monocytes – no significant difference between the two groups (p-value = 0.69).

Eosinophils – no significant difference between the two groups (p-value = 0.24).

2.8 Lavage Protein Concentration

A non-parametric hypothesis test is used because of the small number of data points used for the comparison with many values close to zero (six for each group).

A two-sample Wilcoxon test showed no significant difference in Lavage Protein concentration between the Air and HD exposure groups (p-value = 0.065).

2.9 Lung Wet Weight: Body Weight Ratio

The F test indicated that the variances for the groups were consistent.

Normal probability plots for the data in the two groups suggested that this approximation was a reasonable assumption.

The two-sample t-test showed no significant difference in Lung Wet Weight between the Air and HD exposure groups (p-value = 0.089).

2.10 Lung Wet Weight to Dry Weight Ratio

Barlett's test showed no significant differences in the variability for the two groups and positions.

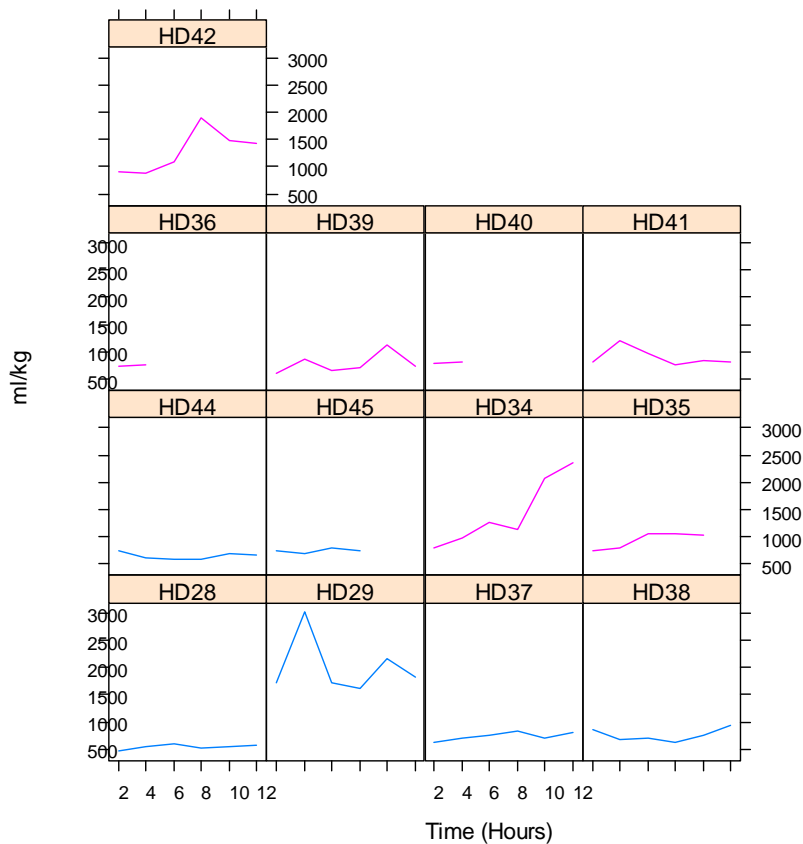
Normal probability plots for the data in the two groups suggested that this approximation was broadly a reasonable assumption.

There is no significant (p-value = 0.08) difference in mean Lung Wet Weight to Dry Weight ratio for the Air control and HD exposure groups

2.11 Extra Vascular Lung Water Content

A graph of Extra Vascular Lung Water against time for each of the animals shows consistency across time for the Air control group (blue lines) in general, except for animal HD29 where there is an unexpected pattern. For the HD exposure group (pink lines) there appears to be a pattern of increasing Extra Vascular Lung Water against time for some but not all of the animals. It should be noted as with the previous measures some of the animals in the HD exposure may have died before an effect could have been observed.

Extra Vascular Lung Water



Bartlett's test indicated that the variances for the groups and time periods were not consistent.

Normal probability plots for the data in the two groups suggested that this approximation was a reasonable assumption at the time points 2, 4, 8 and 12 hours.

Air control versus HD exposure group comparisons:

At 2 hours there is no significant difference between the two groups (p-value = 0.613).

At 4 hours there is no significant difference between the two groups (p-value = 0.725).

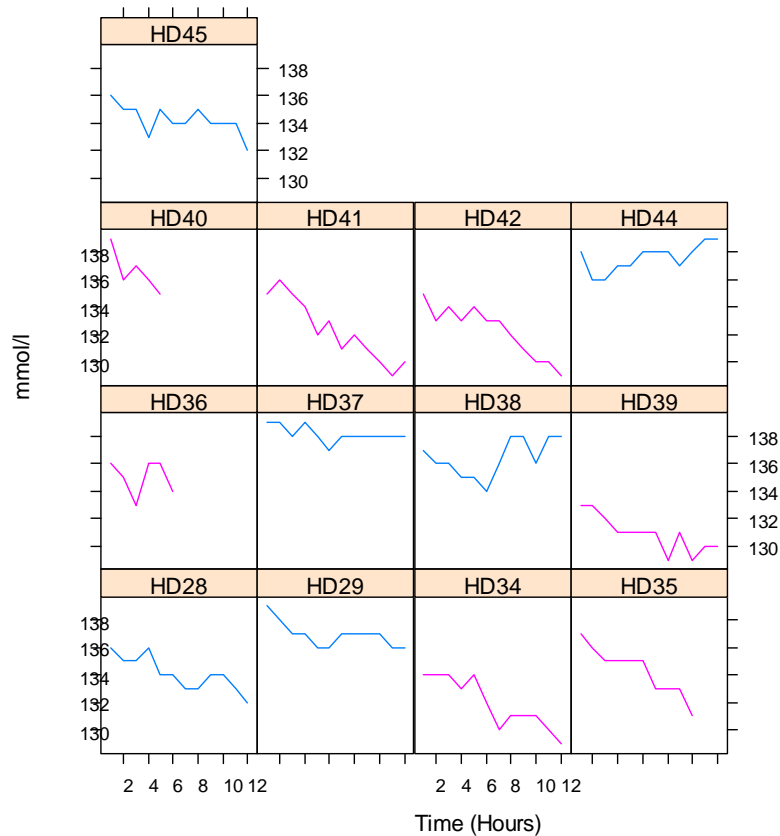
At 8 hours there is no significant difference between the two groups (p-value = 0.320).

At 12 hours there is no significant difference between the two groups (p-value = 0.456).

2.12 Arterial Blood Sodium Concentration

A graph of the data plotted for individual animals indicates that the Arterial Blood Sodium concentration shows no general trend for the Air control group (blue lines) and there is a general trend of a decrease for the HD exposure group (pink lines):

Arterial Blood Sodium Concentration



Bartlett's test shows the variability for the two groups is consistent at 3, 6 and 9 hours but is different at 12 hours.

Normal probability plots for the data in the two groups suggested that this approximation was broadly a reasonable assumption at the time points 3, 6, 9 and 12 hours.

Air control versus HD exposure group comparisons:

At 3 hours there is a significant difference between the two groups (p-value = 0.03).

At 6 hours there is a significant difference between the two groups (p-value = 0.02).

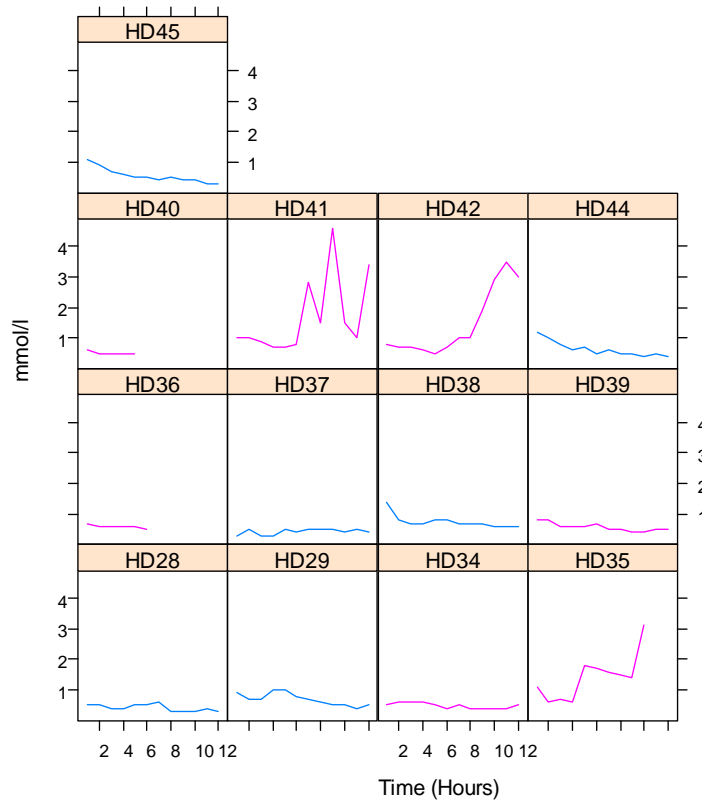
At 9 hours there is a significant difference between the two groups (p-value = 0.0006).

At 12 hours there is a significant difference between the two groups (p-value = 0.004).

2.13 Arterial Blood Lactate Concentration

A graph of the data plotted for individual animals indicates that the Arterial Blood Lactate concentration stays consistent for the Air control group (blue lines) and there is no general trend for the HD exposure group (pink lines) with one animal showing a large amount of variability over time:

Arterial Blood Lactate by Animal



Bartlett's test shows the variability for the two groups is consistent at 6, 9 and 12 hours but is different at 3 hours.

Normal probability plots for the data in the two groups suggested that this approximation was broadly a reasonable assumption at the time points 3, 6, 9 and 12 hours.

Air control versus HD exposure group comparisons:

At 3 hours there is no significant difference between the two groups (p-value = 0.56).

At 6 hours there is no significant difference between the two groups (p-value = 0.32).

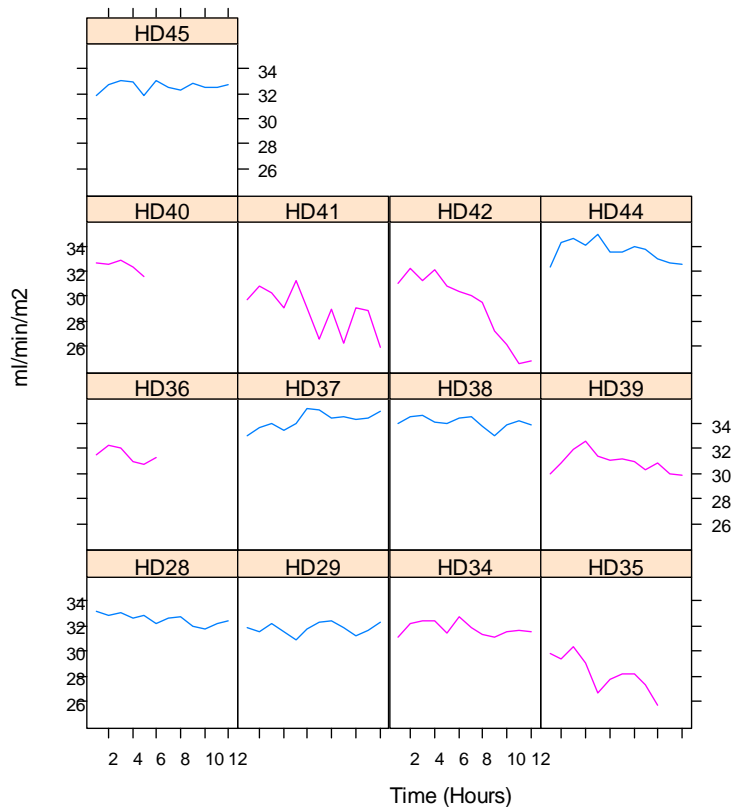
At 9 hours there is no significant difference between the two groups (p-value = 0.18).

At 12 hours there is no significant difference between the two groups (p-value = 0.16).

2.14 Arterial Blood Bicarbonate Concentration

A graph of the data plotted for individual animals indicates that the Arterial Blood Bicarbonate concentration stays consistent for the Air control group (blue lines) and there is some evidence of a general downward trend for the HD exposure group (pink lines):

Arterial Blood Bicarbonate by Anima



Bartlett's test shows the variability for the two groups is consistent at 3, 6, 9 and 12 hours.

Normal probability plots for the data in the two groups suggested that this approximation was broadly a reasonable assumption at the time points 3, 6, 9 and 12 hours.

Air control versus HD exposure group comparisons:

At 3 hours there is a significant difference between the two groups (p-value = 0.004).

At 6 hours there is a significant difference between the two groups (p-value = 0.008).

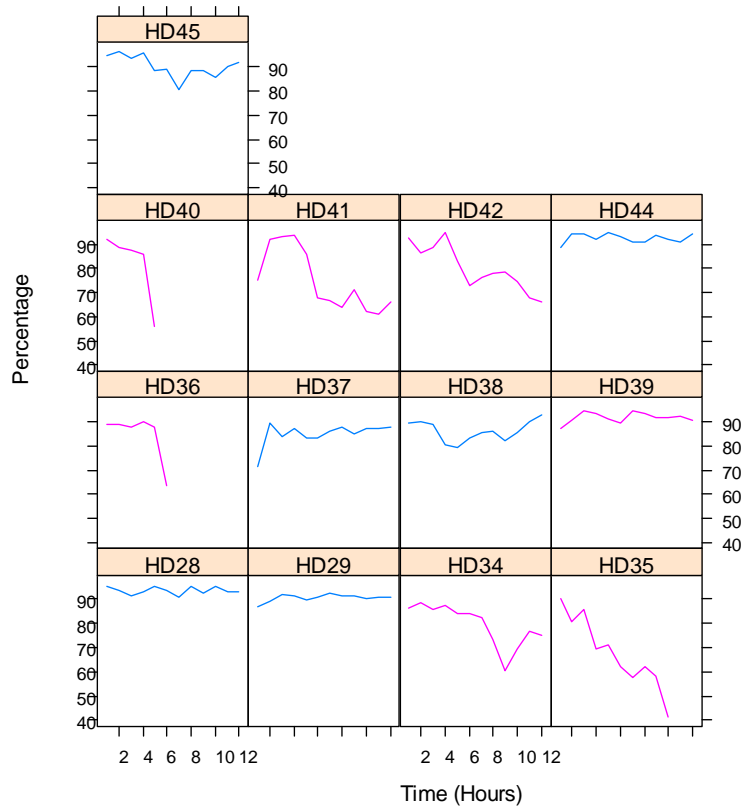
At 9 hours there is a significant difference between the two groups (p-value = 0.006).

At 12 hours there is a significant difference between the two groups (p-value = 0.04).

2.15 Arterial Blood Oxyhaemoglobin

A graph of the data plotted for individual animals indicates that the Arterial Blood Oxyhaemoglobin concentration stays consistent for the Air control group (blue lines) and there is a general trend of a decrease for the HD exposure group (pink lines):

Arterial Blood Oxyhaemoglobin by A



Bartlett's test shows the variability for the two groups is consistent at 3 and 6 hours but is different at 9 and 12 hours.

Normal probability plots for the data in the two groups suggested that this approximation was broadly a reasonable assumption at the time points 3, 6, 9 and 12 hours.

Air control versus HD exposure group comparisons:

At 3 hours there is no significant difference between the two groups (p-value = 0.44).

At 6 hours there is a significant difference between the two groups (p-value = 0.02).

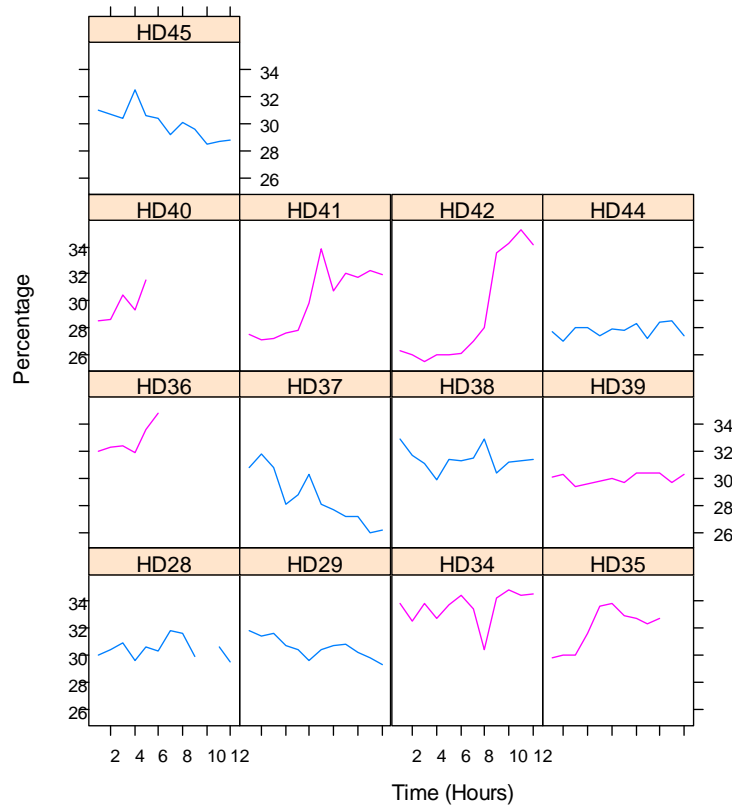
At 9 hours there is a significant difference between the two groups (p-value = 0.05).

At 12 hours there is a significant difference between the two groups (p-value = 0.05).

2.16 Arterial Blood Hematocrit

A graph of the data plotted as individual animals indicates that for Arterial Blood Hematocrit there is no general trend for the Air control group (blue lines) or for the HD exposure group (pink lines):

Arterial Blood Hematocrit by Animal



Bartlett's test shows the variability for the two groups is consistent at 3, 9 and 12 hours but is different at 6 hours.

Normal probability plots for the data in the two groups suggested that this approximation was broadly a reasonable assumption at the time points 3, 6, 9 and 12 hours.

Air control versus HD exposure group comparisons:

At 3 hours there is no significant difference between the two groups (p-value = 0.60).

At 6 hours there is no significant difference between the two groups (p-value = 0.34).

At 9 hours there is a significant difference between the two groups (p-value = 0.006).

At 12 hours there is a significant difference between the two groups (p-value = 0.02).

Conclusion

The variability of the data was not consistent for the two groups or at the various time points.

Differences between the Air control group and the HD exposure group were observed in the following situations:

At 6, 9 and 12 hours for Arterial Blood Oxygenation.

At 3, 6, 9 and 12 hours for Arterial Base Excess.

At 6 hours for Shunt Fraction.

At 6 and 12 hours in Respiratory Rate.

At 3, 6, 9 and 12 hours for Arterial Blood Sodium Concentration.

At 3, 6, 9 and 12 hours for Arterial Bicarbonate Concentration.

At 6, 9 and 12 hours for Arterial Blood Oxyhemoglobin

At 9 and 12 hours for Arterial Blood Hematocrit.

There were no observed differences in the other parameters measured.

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ANNEX E Pathology Report



Histopathology

Your Ref: 180kp HDP 2, 3, 4, 9, 13, 14, 18, 19, 21,
6, 12, 15, 22, 24, 7, 8, 20, 26, 27
Our Ref: 09/41410 - 09/41444

Sender: Chris Taylor

Location DSTL Porton Down
: Salisbury, Wiltshire SP4 0JQ

Date: 19th November 2009

Gross Observations

The first study involves pathology assessment of lung injury in an anaesthetised porcine model of sulphur mustard (HD) inhalation. There are a **total of 133 slides** consisting of H&E stained trachea and lung tissue sections from right and left apical, medial and caudal lobes. The right medial lobes have been lavaged so this will likely have a bearing on the observed pathology. Some of the caudal lobe slides have two sections on them from lung areas affected and unaffected by hyperstatic congestion.

The animal codes written on the slides for the separate groups are as follows:

HD 2, 3, 4 & 9 (air controls)
HD 13, 14, 18, 19, 23 (HD dose 1)
HD 6, 12, 15, 22, 24 (HD dose 2)
HD 7, 8, 20, 26, 27 (HD dose 3)

A scoring system has been developed to assess the lung pathology. The parameters that have been measured are the following:

Bronchiolar epithelial degeneration and necrosis

Hypertrophy/Hyperplasia of bronchiolar epithelium

Cell debris in the bronchiolar lumen

Perivascular (and peribronchiolar) oedema, fibrin and cell infiltrates

Alveolar oedema and fibrin

Alveolar cell infiltrates

Interstitial (septal) oedema

Haemorrhages

Parameters were evaluated as follows:

0: Not observed

1: Mild

2: Moderate

3: Severe

Results are summarised in the attached Table

WNL: *Within Normal Limits*

Microscopic Observations

Results are summarised in the attached Table

Other findings observed in individual animals (lung pathology) are:

HD2 RA, LC, RC. Artefactual areas of atelectasia

HD3 LC, RC. Artefactual areas of atelectasia

HD4 LC. Artefactual areas of atelectasia

HD 9 LA. Artefactual areas of atelectasia

HD18 LA. Artefactual areas of atelectasia

HD21 LA, LC, RC. Artefactual areas of atelectasia

HD6 LC, RC. Artefactual areas of atelectasia

HD22 RC. Artefactual areas of atelectasia

HD7 LC, RC. Artefactual areas of atelectasia

HD 20 LA, LC. Artefactual areas of atelectasia

HD 26 LA, RC. Artefactual areas of atelectasia

HD 27 LC. Artefactual areas of atelectasia

Trachea:

HD2. Inflammatory cell plug attached to the mucosa (mostly neutrophils) with bacterial colonies intralesional

HD3. WNL

HD4 Small plug of inflammatory cells (neutrophils) and mucus

HD9. WNL

HD13. WNL

HD14. WNL

HD18. Minimal area with neutrophils in the mucosa

HD19. Mild epithelial necrosis and congestion

HD21. WNL

HD6. WNL

HD12. Congestion

HD15. WNL

HD22. WNL

HD24. WNL

HD7. WNL

HD8. No sample

HD20. WNL

HD26. WNL

HD27. WNL

Diagnosis

Lesions induced are minimal or only mild. The most relevant are mild necrosis of the epithelium, presence of cell debris in the bronchiolar lumen, septal and perivascular oedema. Lesions of the trachea can be related to intubation.

Remarks

No samples provided from RA lobe (HD15) and Trachea (HD8)

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Animal	HD 2						HD 3						HD 4						HD 9					
Group	AC						AC						AC						AC					
<i>Lung lobe</i>	LA	RA	LM	RM	LC	RC	LA	RA	LM	RM	LC	RC	LA	RA	LM	RM	LC	RC	LA	RA	LM	RM	LC	RC
Brochiolar epithelial degeneration and necrosis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hypertrophy/Hyperplasia of bronchiolar epithelium	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cell debris in bronchiolar lumen	0	1	0	0	1	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0	1	0	0	0
Perivascular oedema, fibrin and cell infiltrates	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	1	1	0	0
Alveolar oedema and fibrin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alveolar cell infiltrates	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Interstitial oedema	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0
Haemorrhages	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Sum	0	1	0	1	1	2	0	0	0	1	1	1	0	0	0	1	2	1	0	1	2	2	1	0

Animal	HD 13						HD 14						HD 18						HD 19						HD 21					
Group	HD dose 1						HD dose 1						HD dose 1						HD dose 1						HD dose 1					
Lung lobe	LA	RA	LM	RM	LC	RC	LA	RA	LM	RM	LC	RC	LA	RA	LM	RM	LC	RC	LA	RA	LM	RM	LC	RC	LA	RA	LM	RM	LC	RC
Brochiolar epithelial degeneration and necrosis	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1	1	1	0	1	1	0	0	0	0	0	0
Hypertrophy/Hyperplasia of bronchiolar epithelium	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	1	0	0	0	0
Cell debris in bronchiolar lumen	1	1	1	1	0	1	1	1	0	1	1	0	1	1	0	0	1	0	0	1	0	1	0	0	1	2	1	0	0	1
Perivascular oedema, fibrin and cell infiltrates	0	0	0	1	2	1	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	1	0	0	0	0	0	0
Alveolar oedema and fibrin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Alveolar cell infiltrates	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Interstitial oedema	1	1	1	1	0	0	0	0	0	2	0	0	0	0	1	3	0	0	0	0	0	1	0	0	0	0	0	0	2	0
Haemorrhages	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	2	1
Sum	3	2	2	3	2	3	2	1	0	3	1	0	1	2	1	4	1	2	2	1	3	2	2	4	3	1	1	2	3	2

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Animal	HD 6						HD 12						HD 15						HD 22						HD 24					
Group	HD dose 2						HD dose 2						HD dose 2						HD dose 2						HD dose 2					
<i>Lung lobe</i>	LA	RA	LM	RM	LC	RC	LA	RA	LM	RM	LC	RC	LA	RA	LM	RM	LC	RC	LA	RA	LM	RM	LC	RC	LA	RA	LM	RM	LC	RC
Brochiolar epithelial degeneration and necrosis	0	0	1	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
Hypertrophy/Hyperplasia of bronchiolar epithelium	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cell debris in bronchiolar lumen	0	0	0	0	1	1	1	1	1	1	0	0	1	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	1	0
Perivascular oedema, fibrin and cell infiltrates	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	1	0	0	1	0	0	0
Alveolar oedema and fibrin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alveolar cell infiltrates	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Interstitial oedema	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	2	0	2	0	0	0	1	0	0	0	0	0	2	0	0
Haemorrhages	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0

Sum 0 0 3 2 2 1 2 2 1 1 2 1 1 NE 1 3 1 3 0 0 1 2 1 2 1 1 0 2 1 1

Animal	HD 7						HD 8						HD 20						HD 26						HD 27					
Group	HD dose 3						HD dose 3						HD dose 3						HD dose 3						HD dose 3					
<i>Lung lobe</i>	LA	RA	LM	RM	LC	RC	LA	RA	LM	RM	LC	RC	LA	RA	LM	RM	LC	RC	LA	RA	LM	RM	LC	RC	LA	RA	LM	RM	LC	RC
Brochiolar epithelial degeneration and necrosis	2	1	0	1	0	0	1	0	1	0	1	1	0	1	2	1	0	0	0	2	1	1	1	0	1	1	1	0	0	1
Hypertrophy/Hyperplasia of bronchiolar epithelium	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cell debris in bronchiolar lumen	0	1	1	0	1	2	1	1	0	1	1	1	1	0	1	1	3	3	1	0	1	0	0	1	1	1	1	0	1	1
Perivascular oedema, fibrin and cell infiltrates	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	1	1	0	1	0	1	1	0	1
Alveolar oedema and fibrin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alveolar cell infiltrates	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Interstitial oedema	0	2	0	2	0	0	2	2	3	1	2	2	0	0	0	0	0	0	0	2	3	2	2	0	2	0	1	2	0	1
Haemorrhages	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0

Sum 3 4 1 3 2 3 4 3 5 2 5 4 2 1 3 3 4 4 1 4 6 4 4 2 5 2 4 3 1 4

Pathologist Signature Name F. Javier Salguero Bodes, DVM, PhD, MRCVS

Date 19th November 2009

ANNEX F Pathology Report



Histopathology

Your Ref: HDP 28, 29, 37, 38, 44, 45, 34, 35, 36,
39, 40, 41 & 42

Our Ref: 10/40054 - 10/40066

Sender: Chris Taylor

Location DSTL. Porton Down
: Salisbury, Wiltshire SP4 0JQ

Date: 29th January 2010

Gross Observations

Pathology assessment of lung injury in an anaesthetised porcine model of sulphur mustard (HD) inhalation. Continuation of previous study. There are a total of 91 slides consisting of H&E stained trachea and lung tissue sections from right and left apical, medial and caudal lobes.

The animal codes written on the slides for the separate groups are as follows:

HDP 28, 29, 37, 38, 44 & 45 (air controls)

HDP 34, 35, 36, 39, 40, 41 & 42 (HDP dose)

A scoring system has been developed to assess the lung pathology. The parameters that have been measured are the following:

- Bronchiolar epithelial degeneration and necrosis
- Hypertrophy/Hyperplasia of bronchiolar epithelium
- Cell debris in the bronchiolar lumen
- Perivascular (and peribronchiolar) oedema, fibrin and cell infiltrates
- Alveolar oedema and fibrin
- Alveolar cell infiltrates
- Interstitial (septal) oedema
- Haemorrhages

Parameters were evaluated as follows:

0: Not observed

1: Mild

2: Moderate

3: Severe

Results are summarised in the attached Table

WNL: Within Normal Limits

Microscopic Observations

Results are summarised in the attached Table

Other findings observed in individual animals (lung pathology) are:

HDP 28 LC. Artefactual areas of atelectasia

HDP 29 LC, RC. Artefactual areas of atelectasia

HDP 37 LC. Artefactual areas of atelectasia

HDP 38 LC, RC. Artefactual areas of atelectasia

HDP 45 LC. Artefactual areas of atelectasia

HDP 34 LC, RC. Artefactual areas of atelectasia

HDP 35 LC, RC. Artefactual areas of atelectasia

HDP 39 LC, RC. Artefactual areas of atelectasia

Trachea:

HDP 28. Focal area of inflammation: Inflammatory cell plug attached to the mucosa (mostly neutrophils)

HDP 29. WNL

HDP 37. WNL

HDP 38. WNL

HDP 44. WNL

HDP 45. WNL

HDP 34. Mild epithelial necrosis and inflammatory cell infiltration

HDP 35. Multifocal areas of inflammation and mild necrosis of the epithelium

HDP 36. Moderate mucosal hyperaemia and submucosal oedema

HDP 39. Focal area of severe inflammation and oedema

HDP 40. Mild subcutaneous inflammatory infiltration (mostly neutrophils)

HDP 41. Focal area of inflammation in the mucosa. Moderate submucosal oedema, hyperaemia and inflammatory cell infiltration. Moderate epithelium necrosis

HDP 42. Mild submucosal hyperaemia and inflammatory cell infiltration

Diagnosis

Lesions induced are minimal or only mild. The most relevant are mild necrosis of the epithelium, presence of cell debris in the bronchiolar lumen, septal and perivascular oedema.

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Scores

Animal	HDP 28						HDP 29						HDP 37						HDP 38						HDP 44						HDP 45																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																
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Pathologist Signature Name F. Javier Salguero Bodes, DVM, PhD, MRCVS

Date 29th January 2010

ANNEX G Measured Parameters

This annex provides a detailed list of all of the parameters that were measured and recorded as part of the 6 hour and 12 hour studies. It also includes all of the data (in graphical form) collected during the two studies, for those parameters that were not included in the main report. No statistical analysis of the data presented here has been performed.

A Glossary of the measured parameters is shown below the table.

	Parameters
Cardiac physiology (PiCCO)	Mean arterial pressure SVR Heart rate (HR) Continuous Cardiac output (PiCCO) Stroke Volume (SV) Stroke Volume Variation (SVV) Pulse Pressure Variation (PPV) dP Max
PiCCO (thermodilution)	Cardiac output CFI GEDV ITBV Extra Vascular Lung Water (EVLW) PVPI GEF
Animal monitoring (Propaq)	Core Temperature Respiratory rate (RR) Heart rate (HR) Expired CO ₂ Pulse oximetry Mean arterial pressure Central venous pressure
Blood Chemistry (arterial and venous)	pH pO ₂ pCO ₂ Sodium Potassium Calcium Glucose Lactate Hematocrit Temperature Hemoglobin Oxygen saturation (SO ₂) Oxyhemoglobin

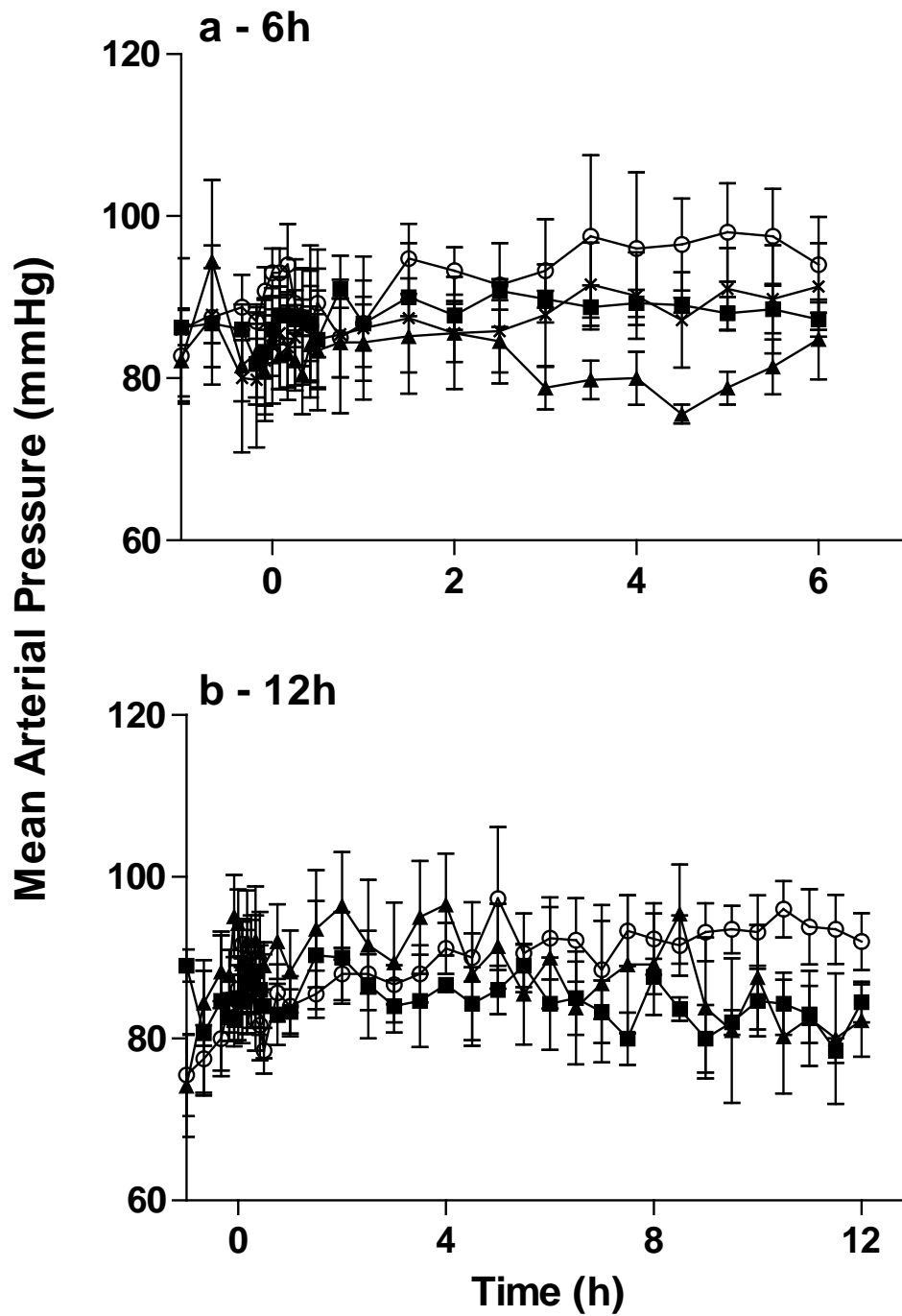
	Carboxyhemoglobin Met Hemoglobin HHb pH temperature corrected pO ₂ temperature corrected pCO ₂ temperature corrected Calcium corrected to pH 7.4 HCO ₃ HCO ₃ std TCO ₂ Base excess (BE) O ₂ ct
Derived cardiovascular variables	Left ventricular stroke work (indexed)
Derived O ₂ transport variables	Oxygen delivery and consumption Alveolar O ₂ content Mixed venous O ₂ content Shunt fraction
Post experiment analysis	Whole blood <ul style="list-style-type: none"> – Haematology – Blood smears Plasma <ul style="list-style-type: none"> – ELISAs (IL-1β, IL-6, CRP, LTB₄, Thromboxane B₂, TNF-α) – Clinical chemistry (parameters listed in Annex B) Lavage <ul style="list-style-type: none"> – Protein concentration – Cell differentials – ELISA (IL-1β, IL-6, IL-8, LTB₄, Thromboxane B₂, TNF-α) Urine <ul style="list-style-type: none"> – Desmosine analysis Tissue <ul style="list-style-type: none"> – Genomics – LWW:DW ratio – LWW:BW ratio

Cardiovascular Parameters

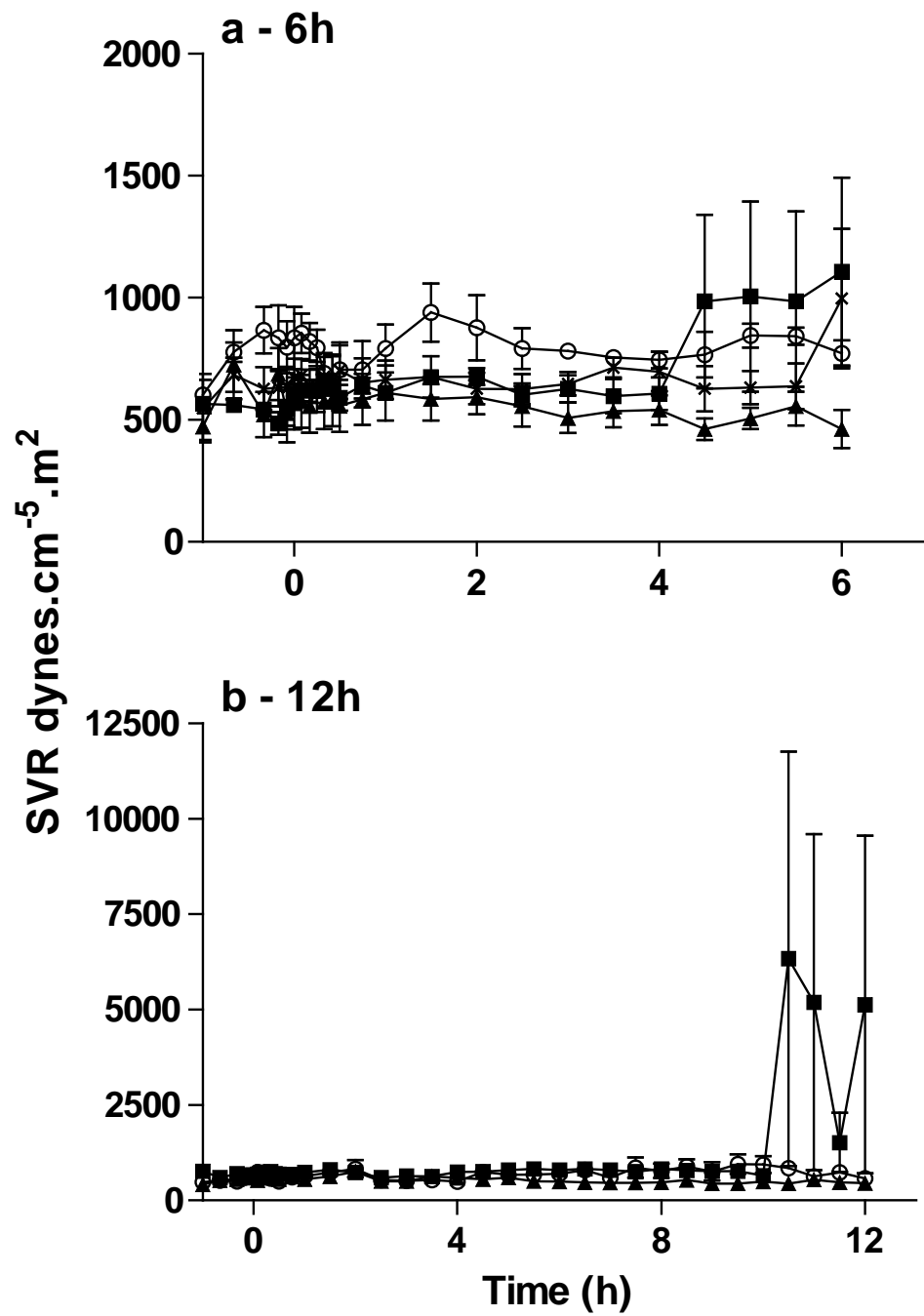
- ~ CFI – Cardiac Function Index
- ~ CO – Cardiac Output
- ~ CvO₂ – Mixed Venous Oxygenation
- ~ CVP – Central Venous Pressure

~	DO ₂	– Oxygen Delivery
~	dpMax	– Maximum diastolic pressure
~	EVLW	– Extravascular Lung Water
~	GEDV	– Global End Diastolic Volume
~	GEF	– Global Ejection Fraction
~	ITBV	– Intra Thoracic Blood Volume
~	LVS _W	– Left Ventricular Stroke Work
~	LWW:BW	– Lung Wet Weight to Body Weight ratio
~	LWW:DW	– Lung Wet Weight to Dry Weight ratio
~	MAP	– Mean Arterial Pressure
~	PAO ₂	– Alveolar Oxygen Concentration
~	PiCCO & CO	– Pulse Contour Cardiac Output
~	PPV	– Pulse Pressure Variation
~	PVPI	– Pulmonary Vascular Permeability Index
~	Q _s :Q _t	– Shunt fraction
~	SV	– Stroke Volume
~	SVR	– Systemic Vascular Resistance
~	SVV	– Stroke Volume Variation
~	V O ₂	– Oxygen Consumption

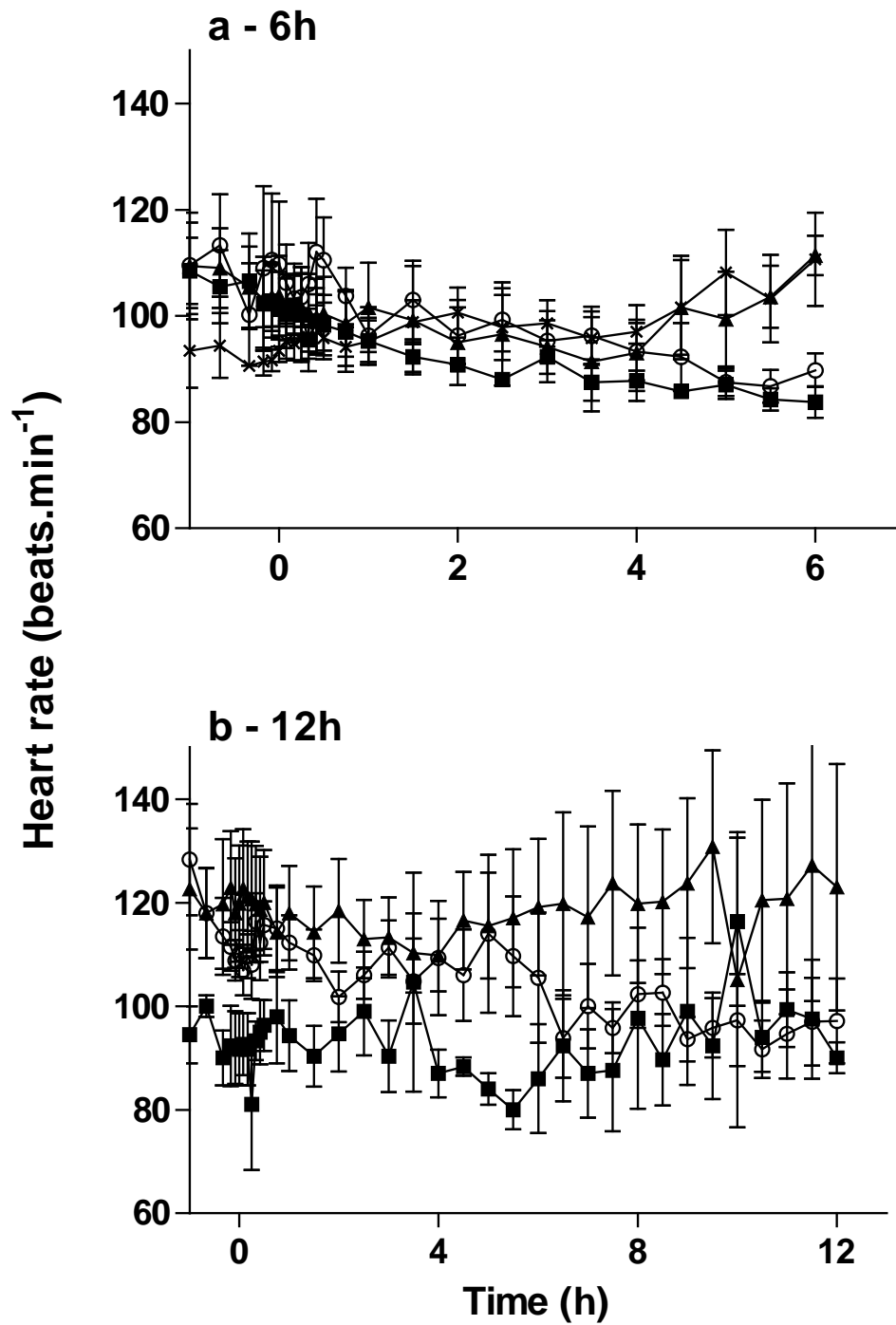
Parameters measured by PiCCO



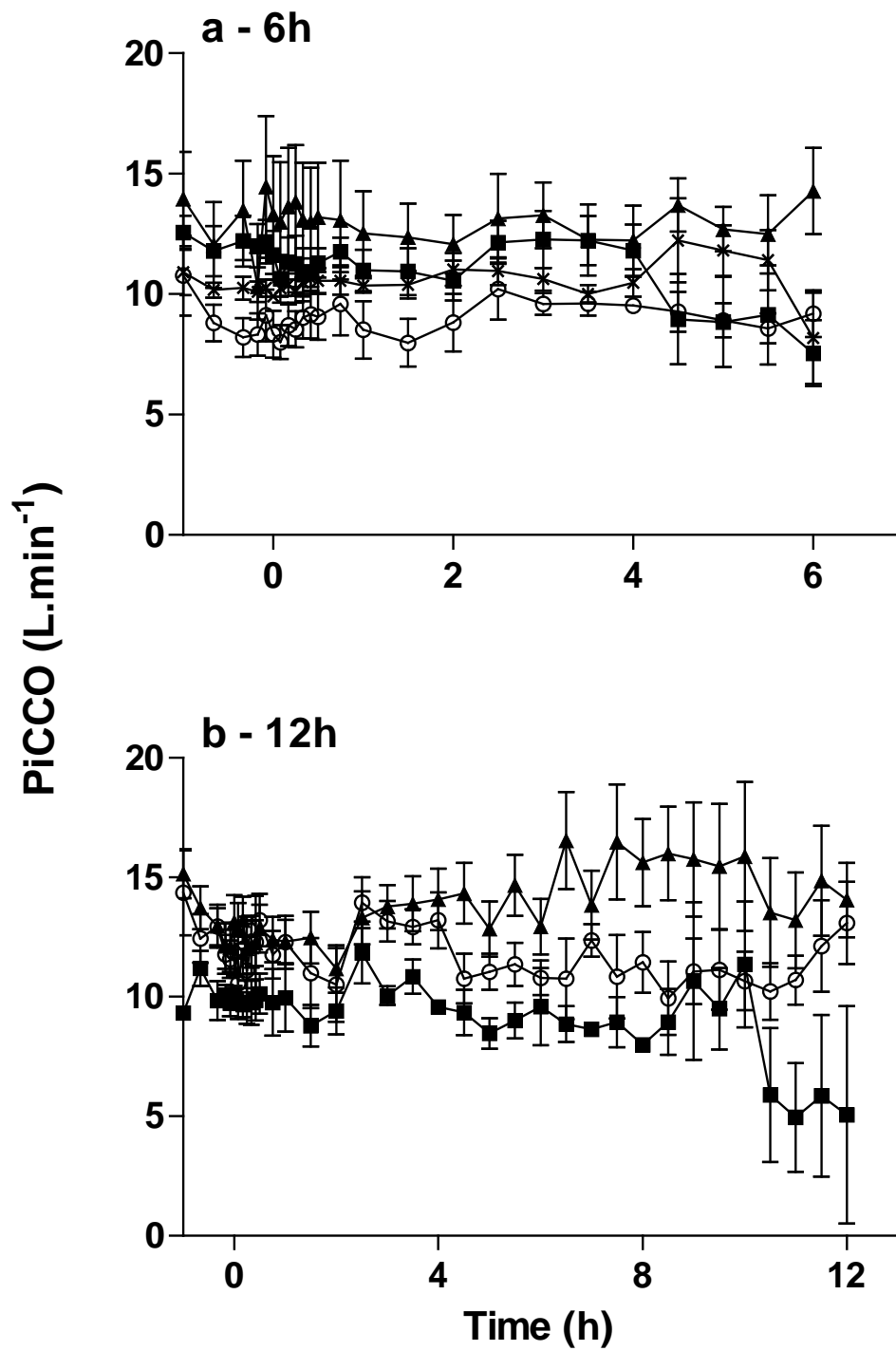
Changes in mean arterial pressure following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



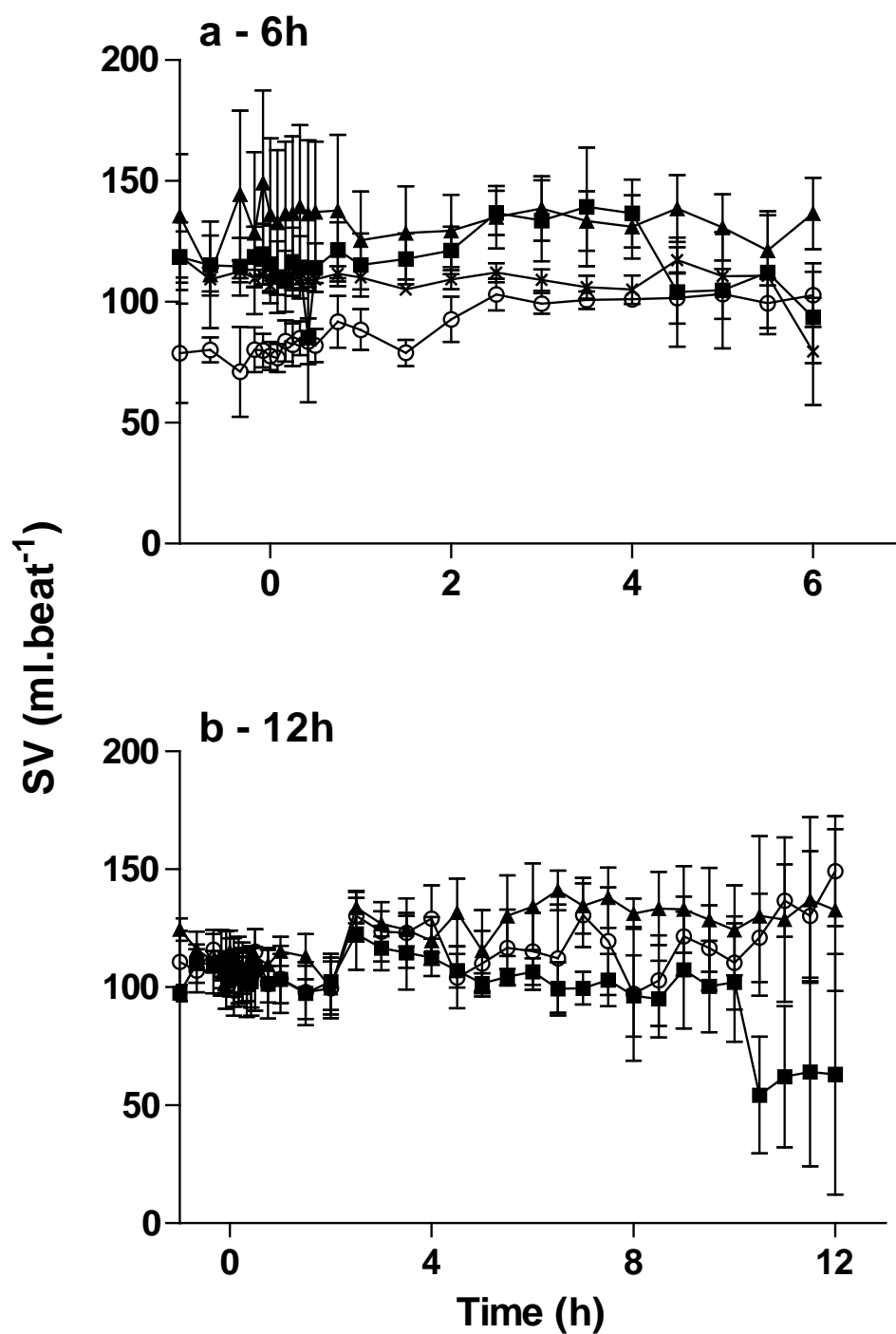
Changes in SVR following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



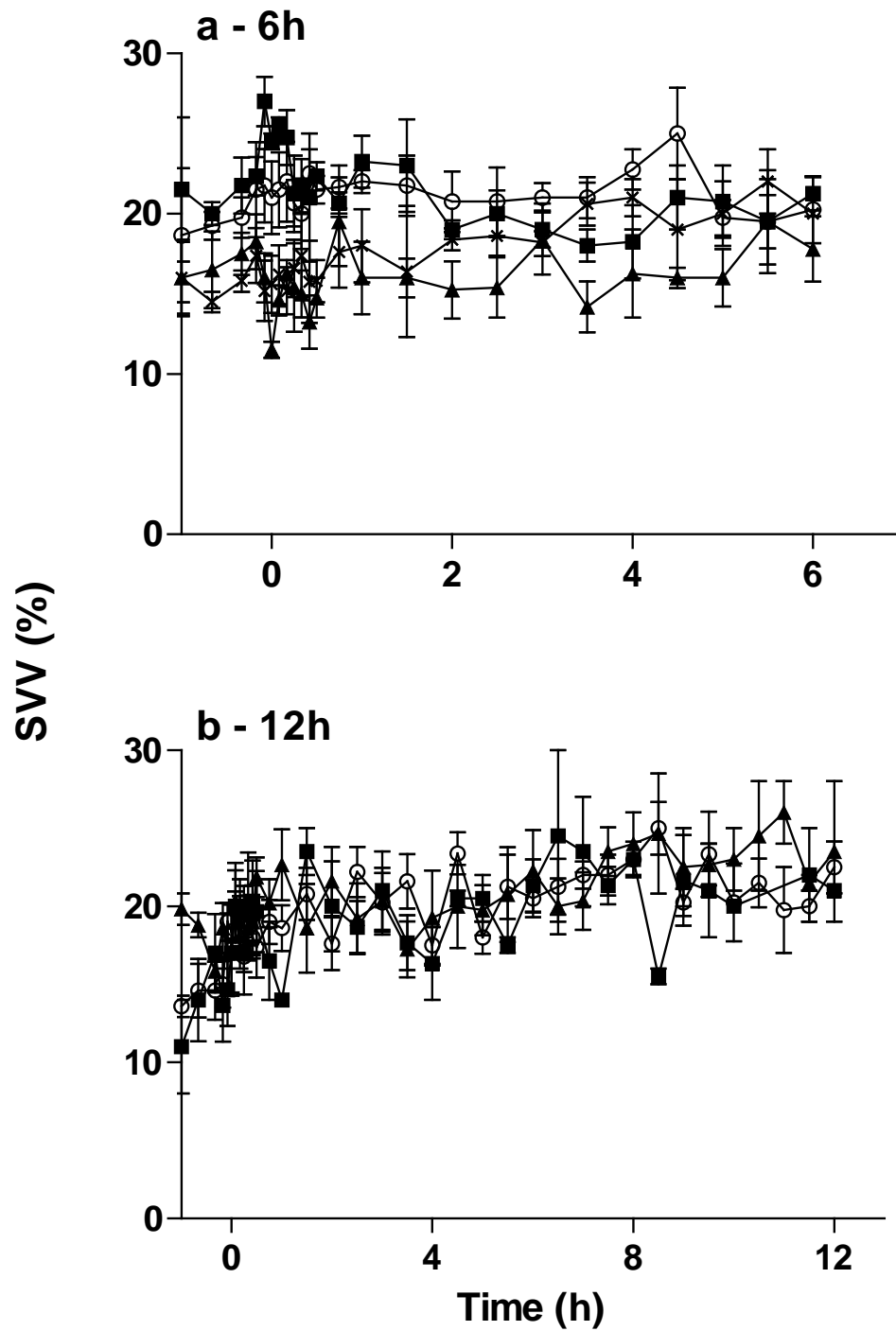
Changes in heart rate measure by PiCCO following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



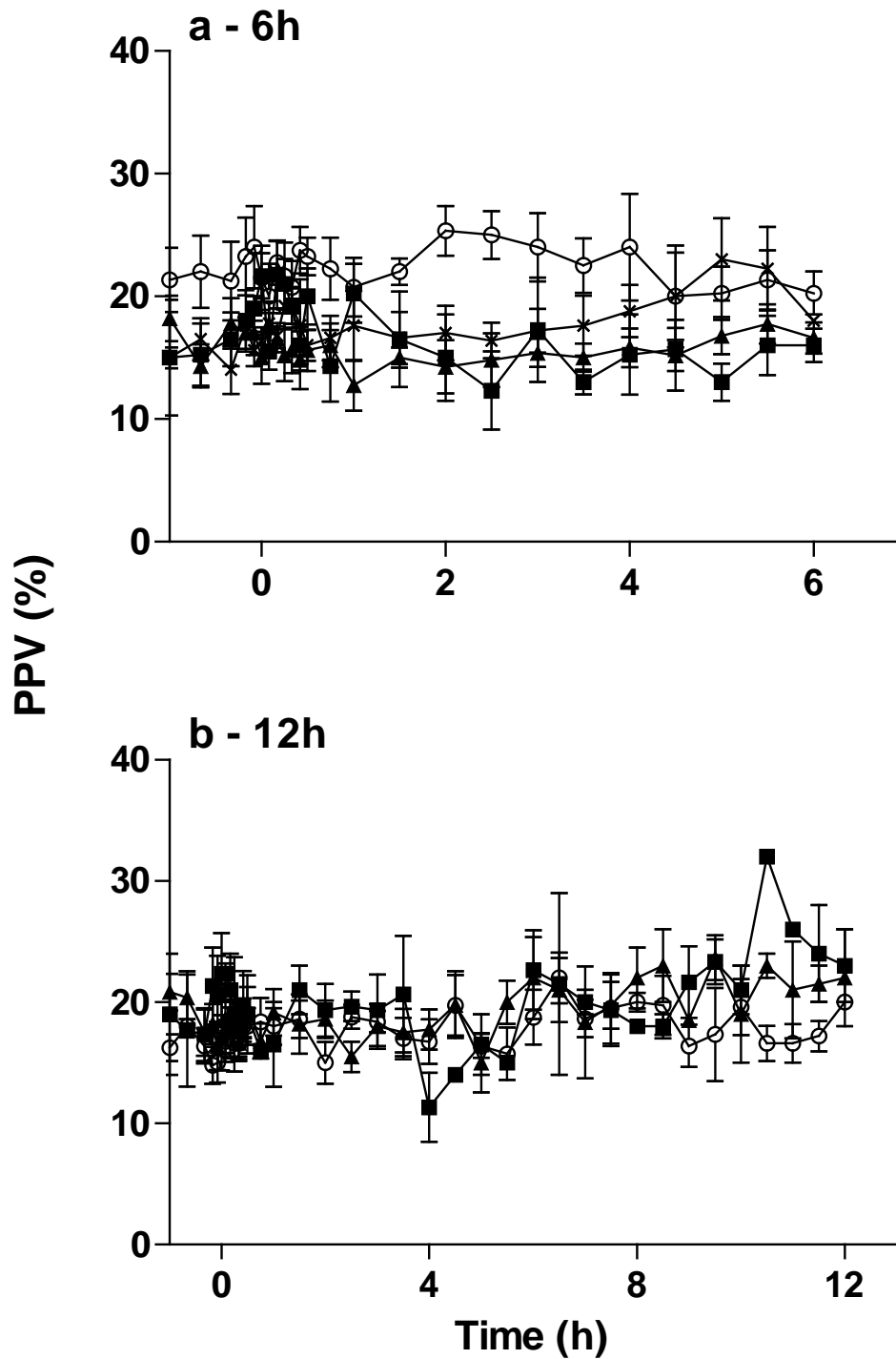
Changes in PiCCO following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



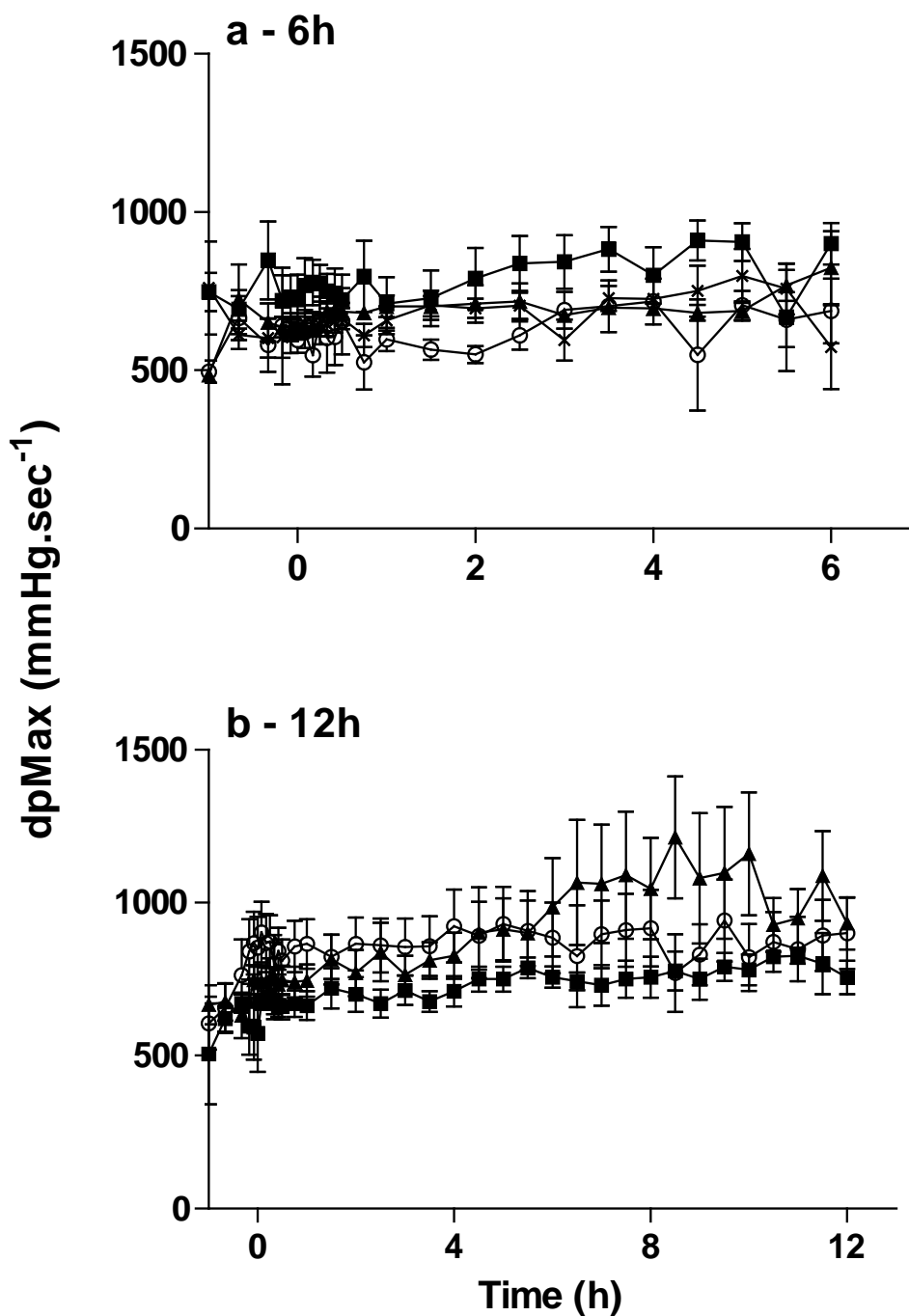
Changes in SV following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (○). Data are expressed as mean \pm SE.



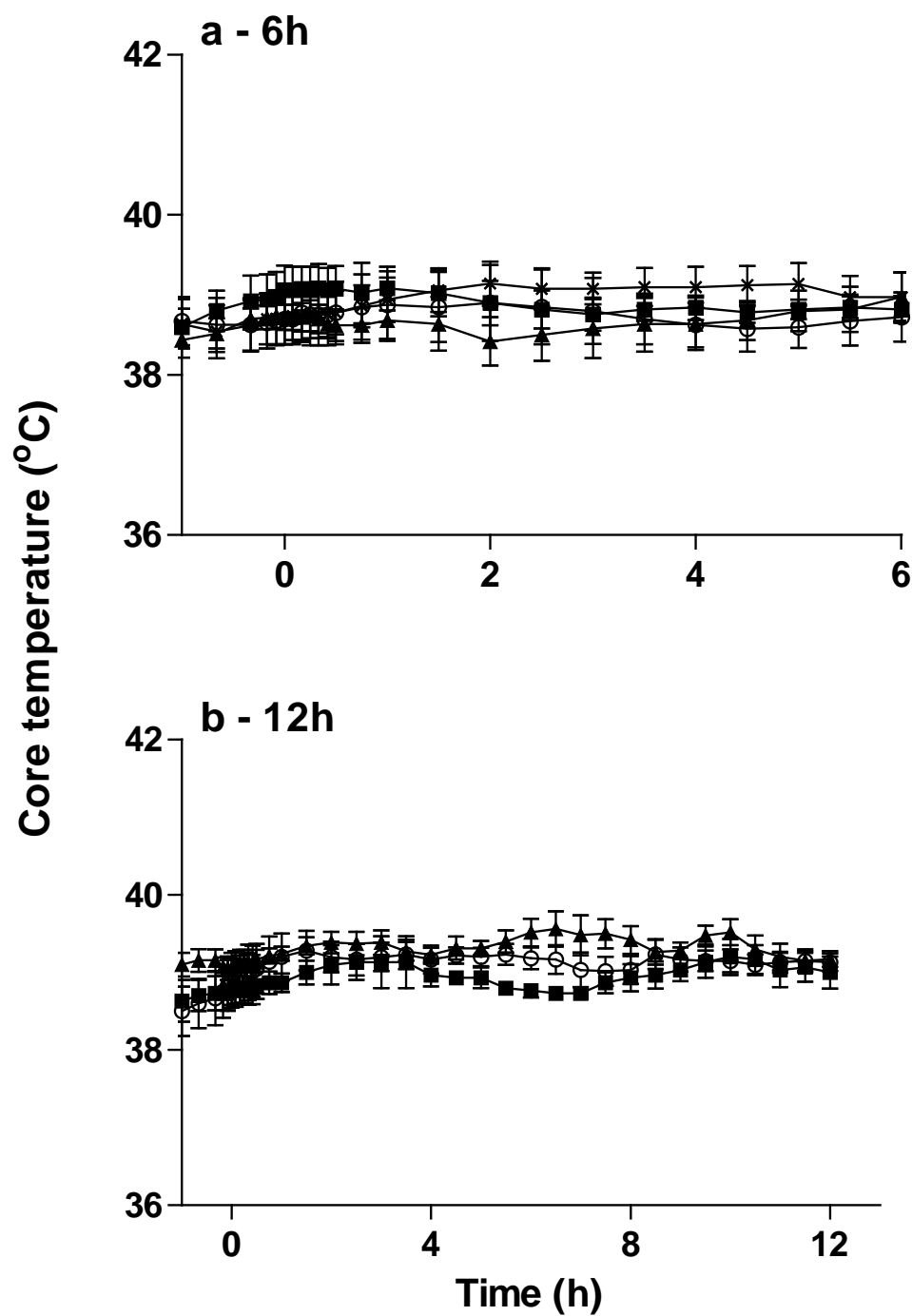
Changes in SVV following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



Changes in PPV following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (○). Data are expressed as mean \pm SE.

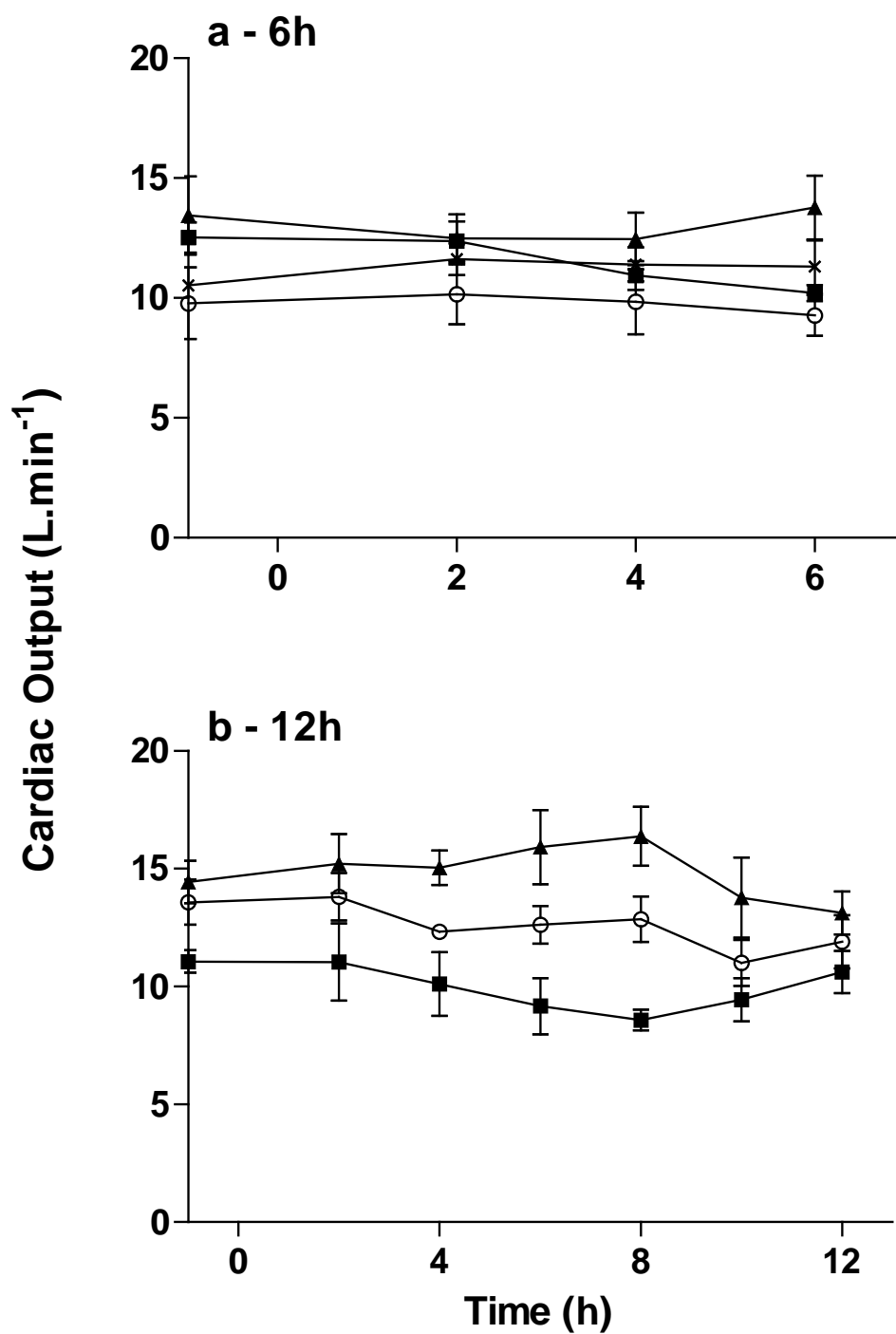


Changes in dpMax following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.

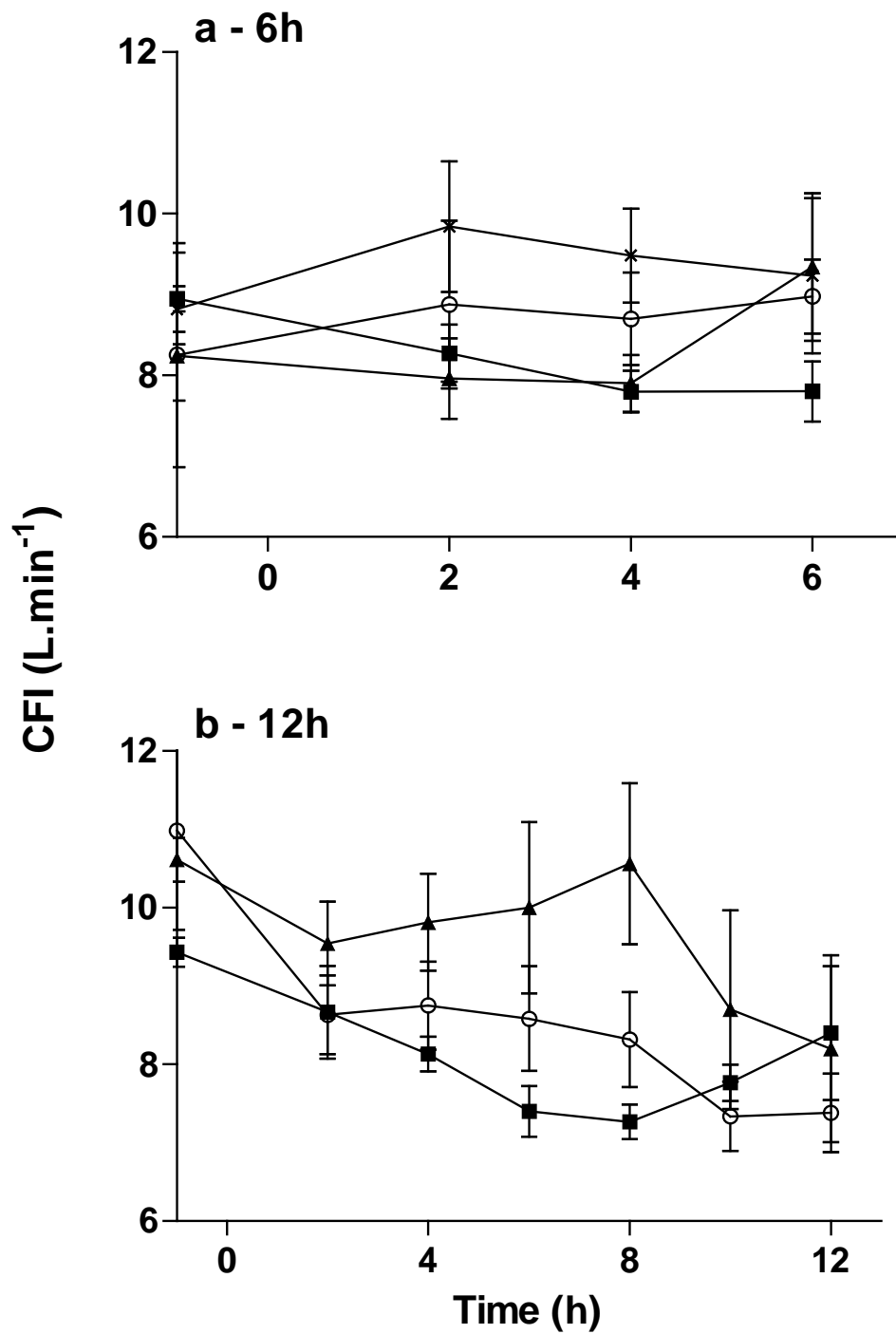


Changes in core temperature measured by PiCCO following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.

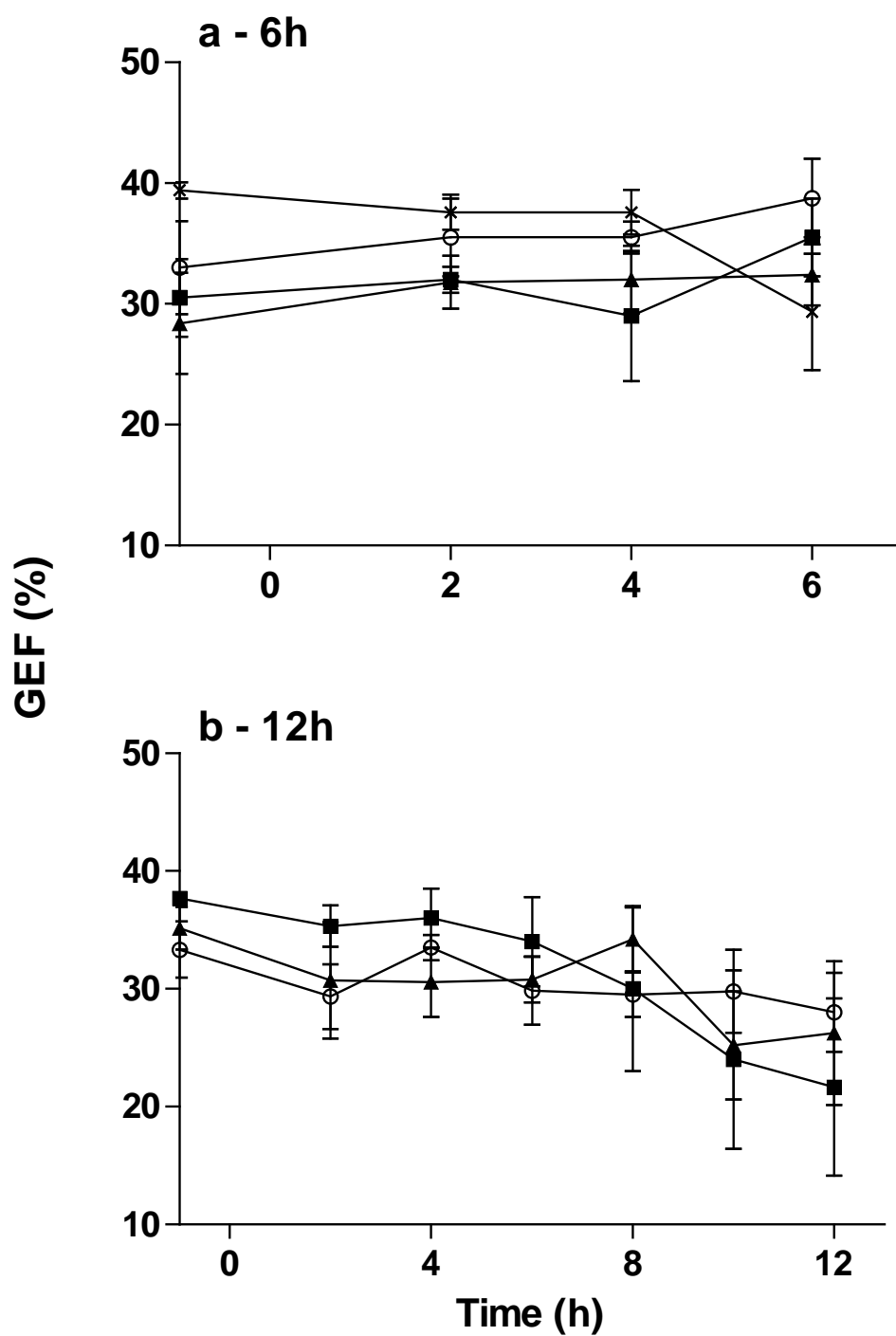
Parameters measured by PiCCO thermodilution



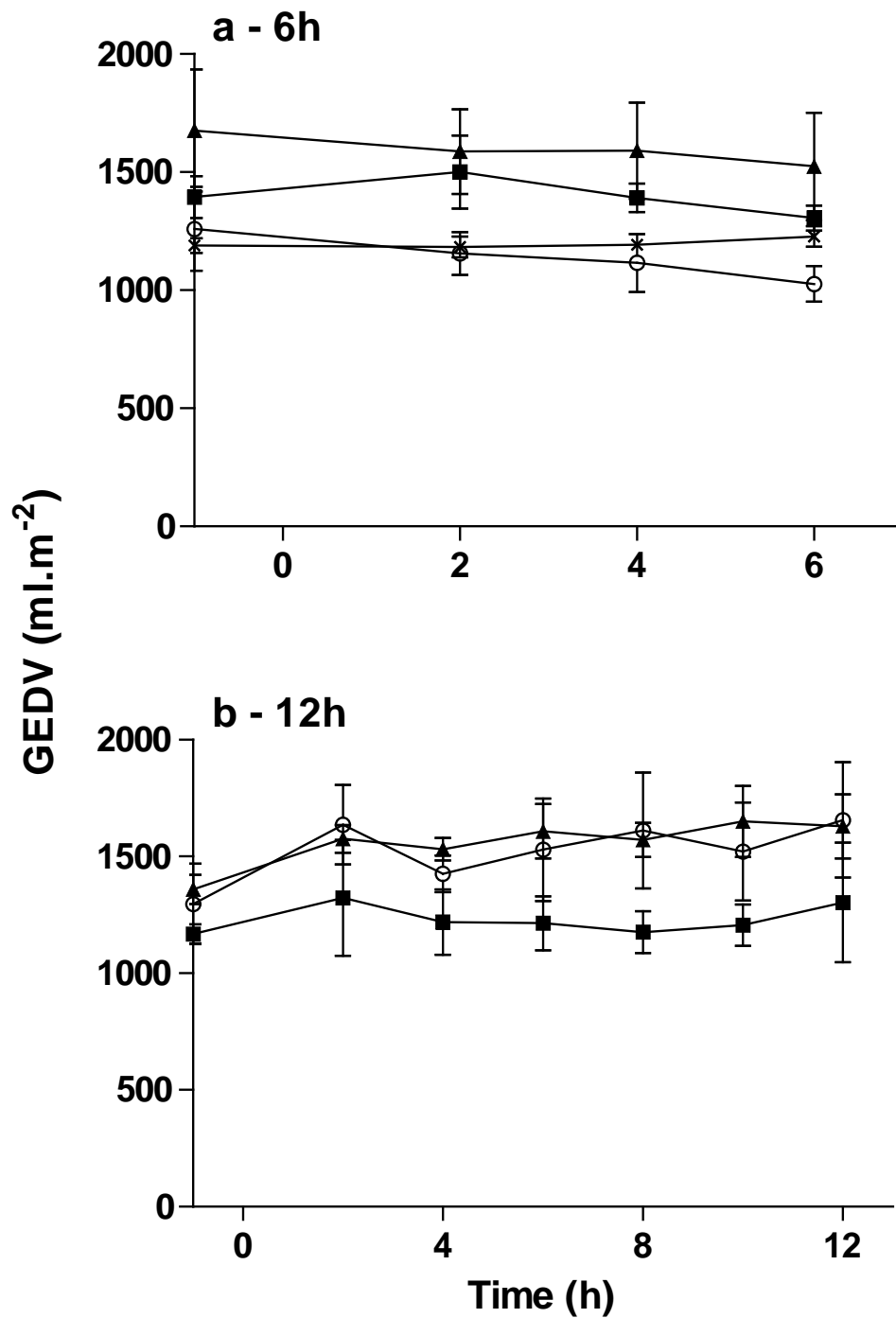
Changes in cardiac output measured by thermodilution following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (○). Data are expressed as mean \pm SE.



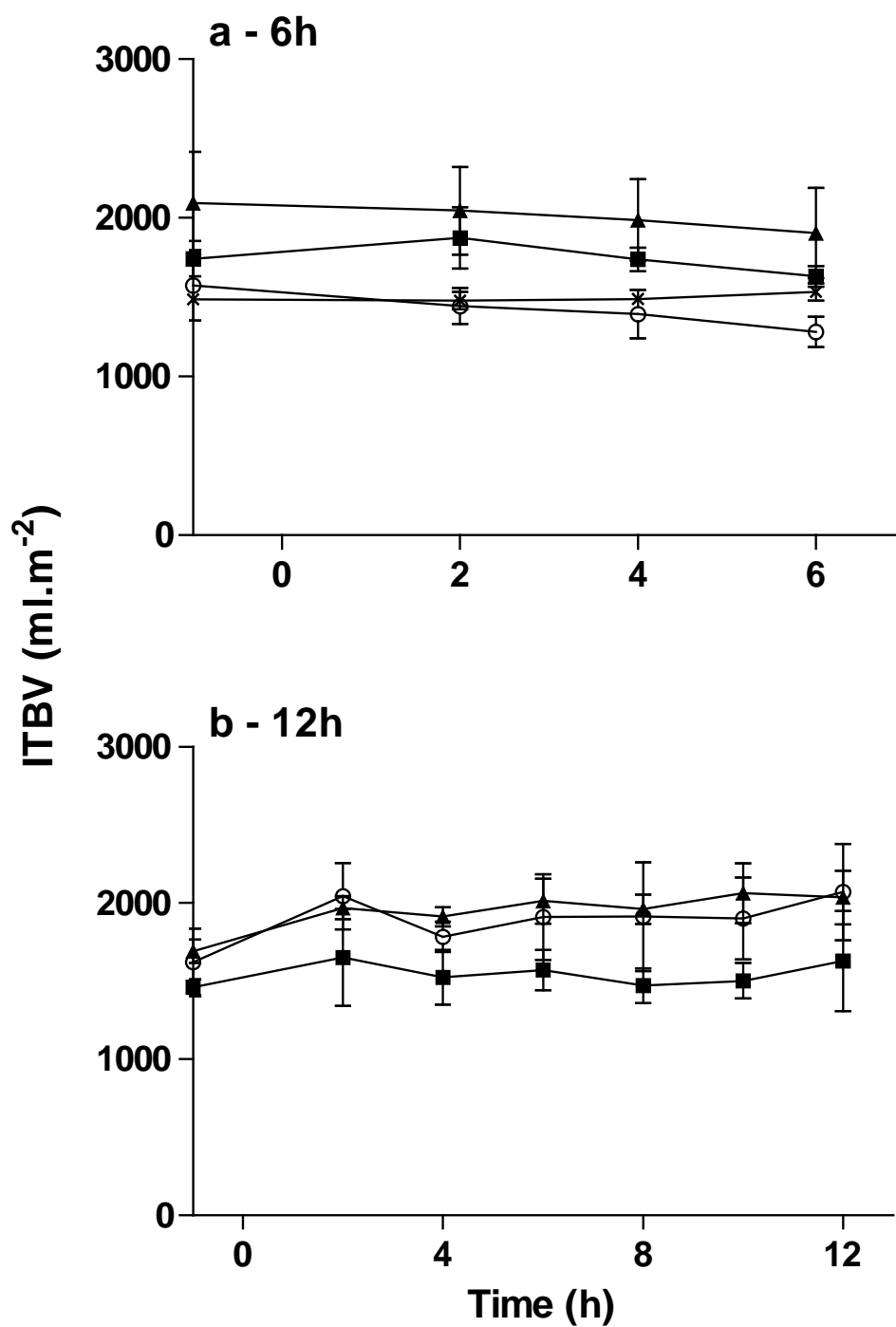
Changes in CFI measured by thermodilution following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



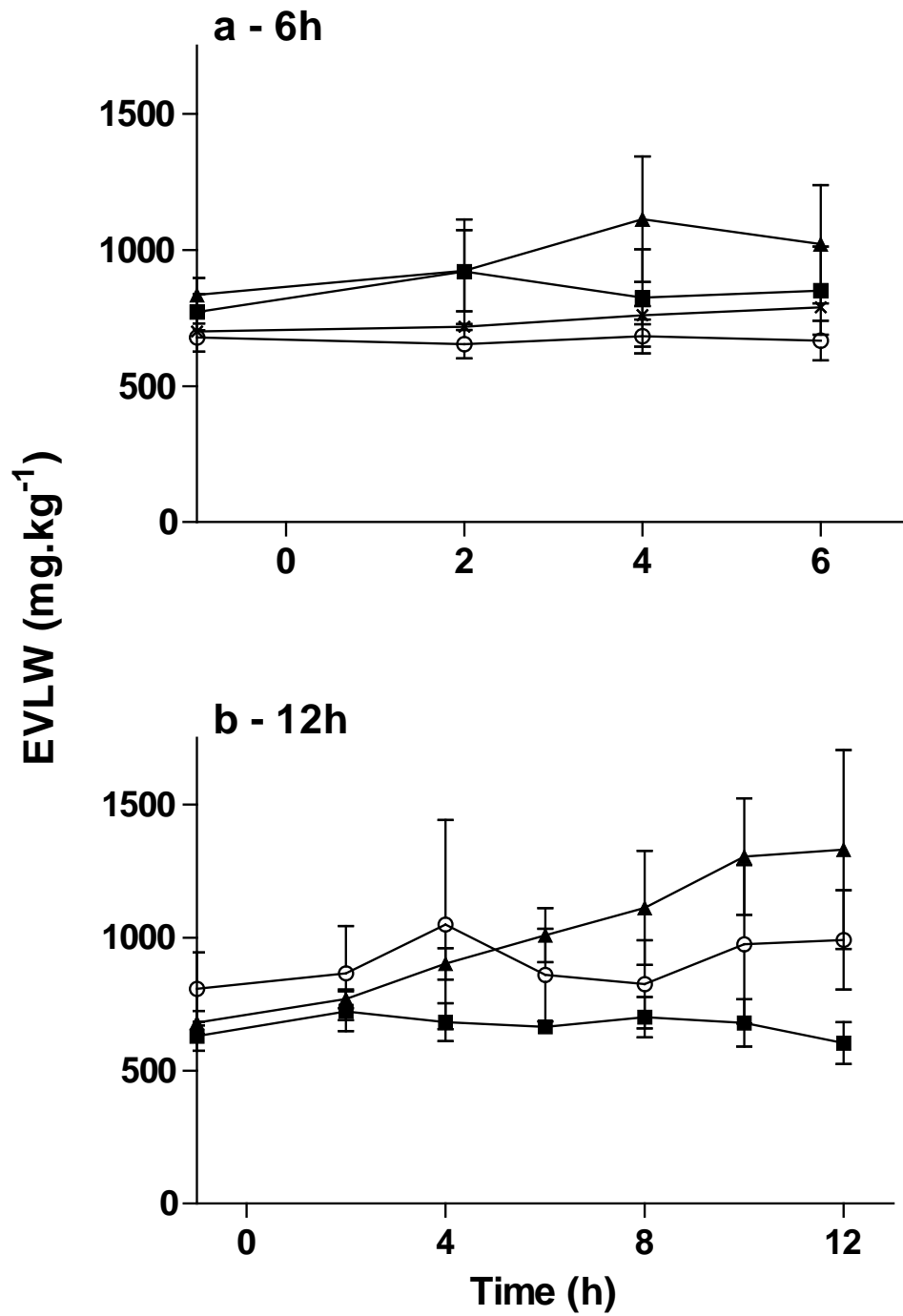
Changes in GEF measured by thermodilution following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



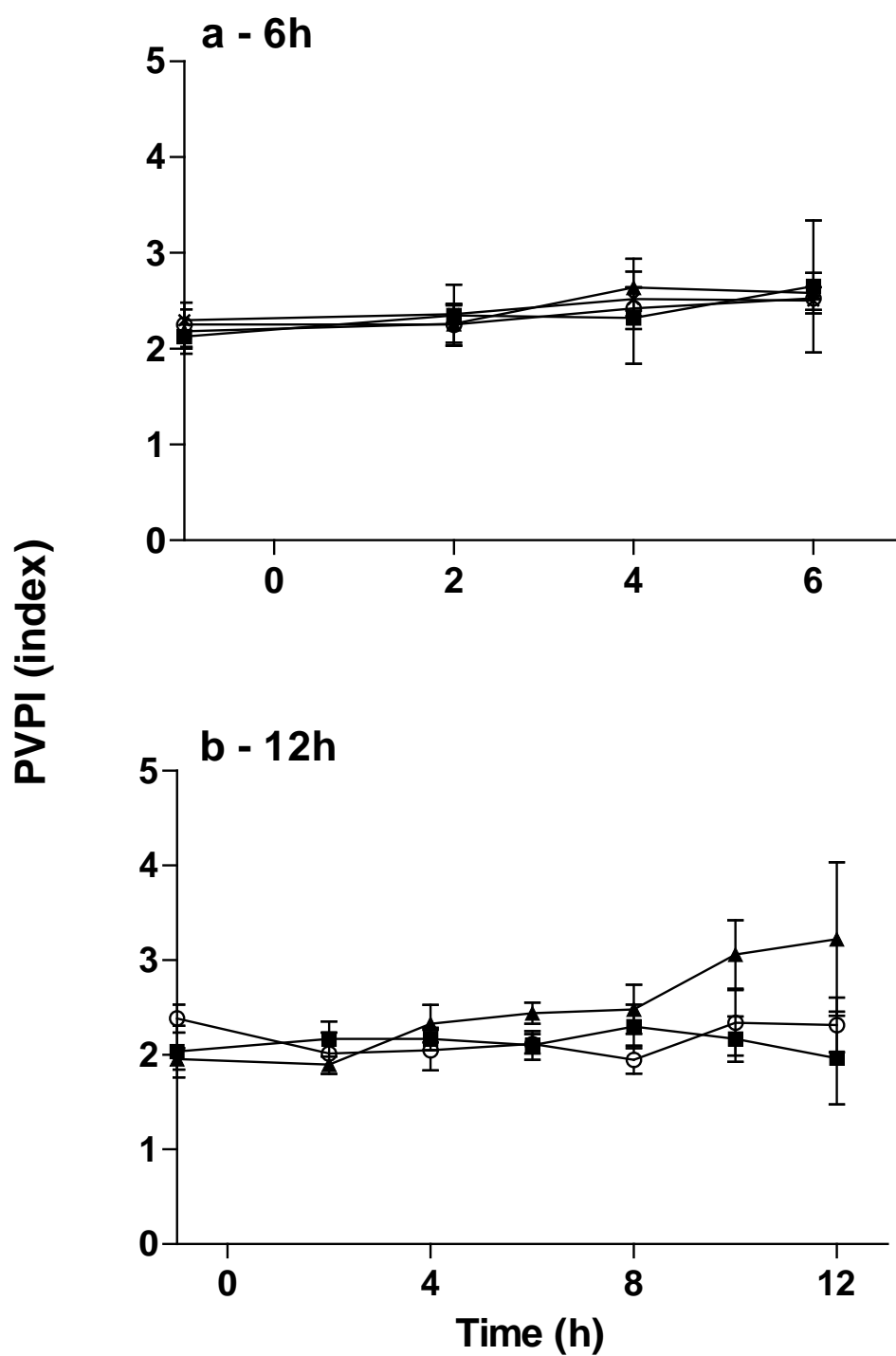
Changes in GEDV by thermodilution following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



Changes in ITBV measured by thermodilution following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.

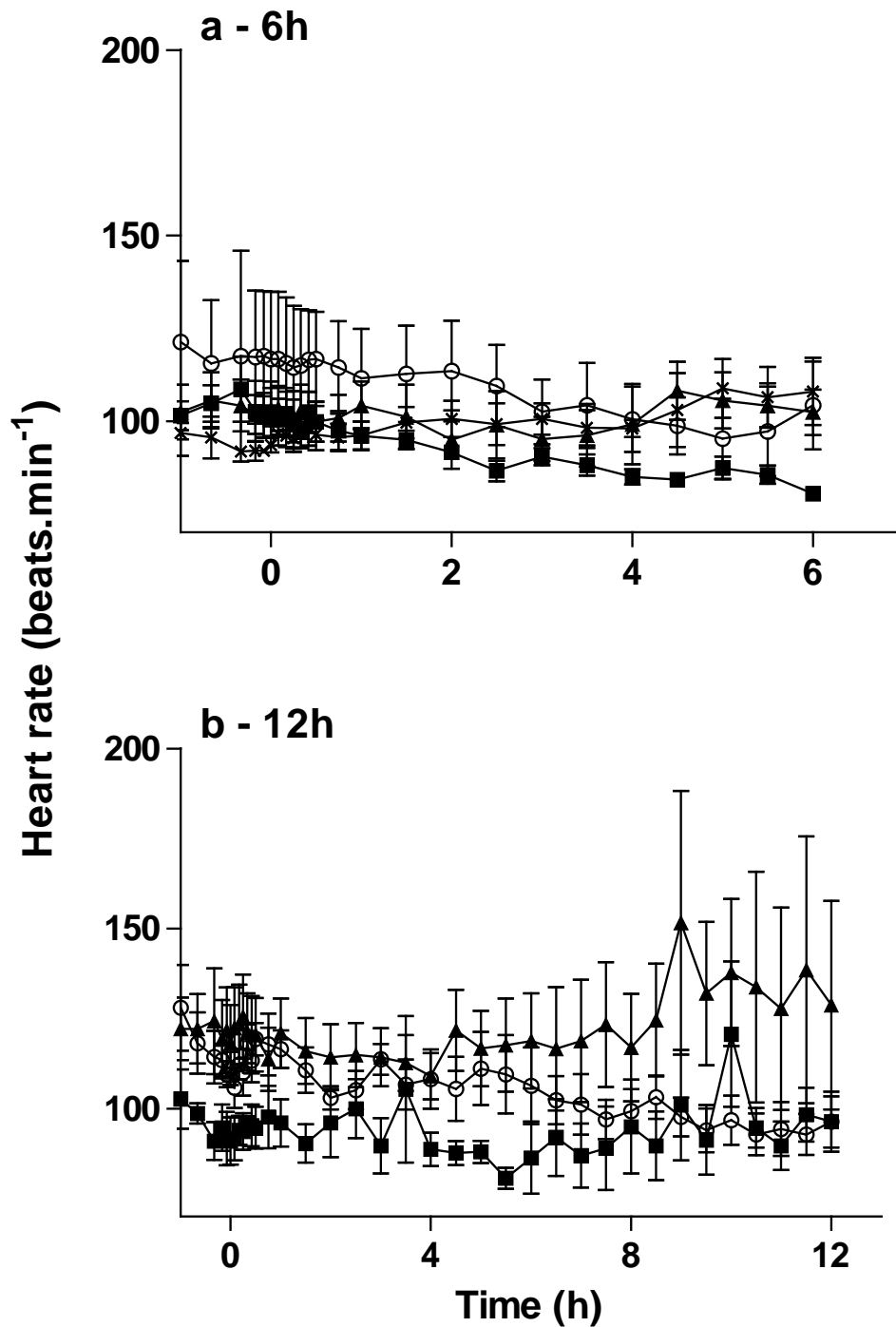


Changes in EVLW measured by thermodilution following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.

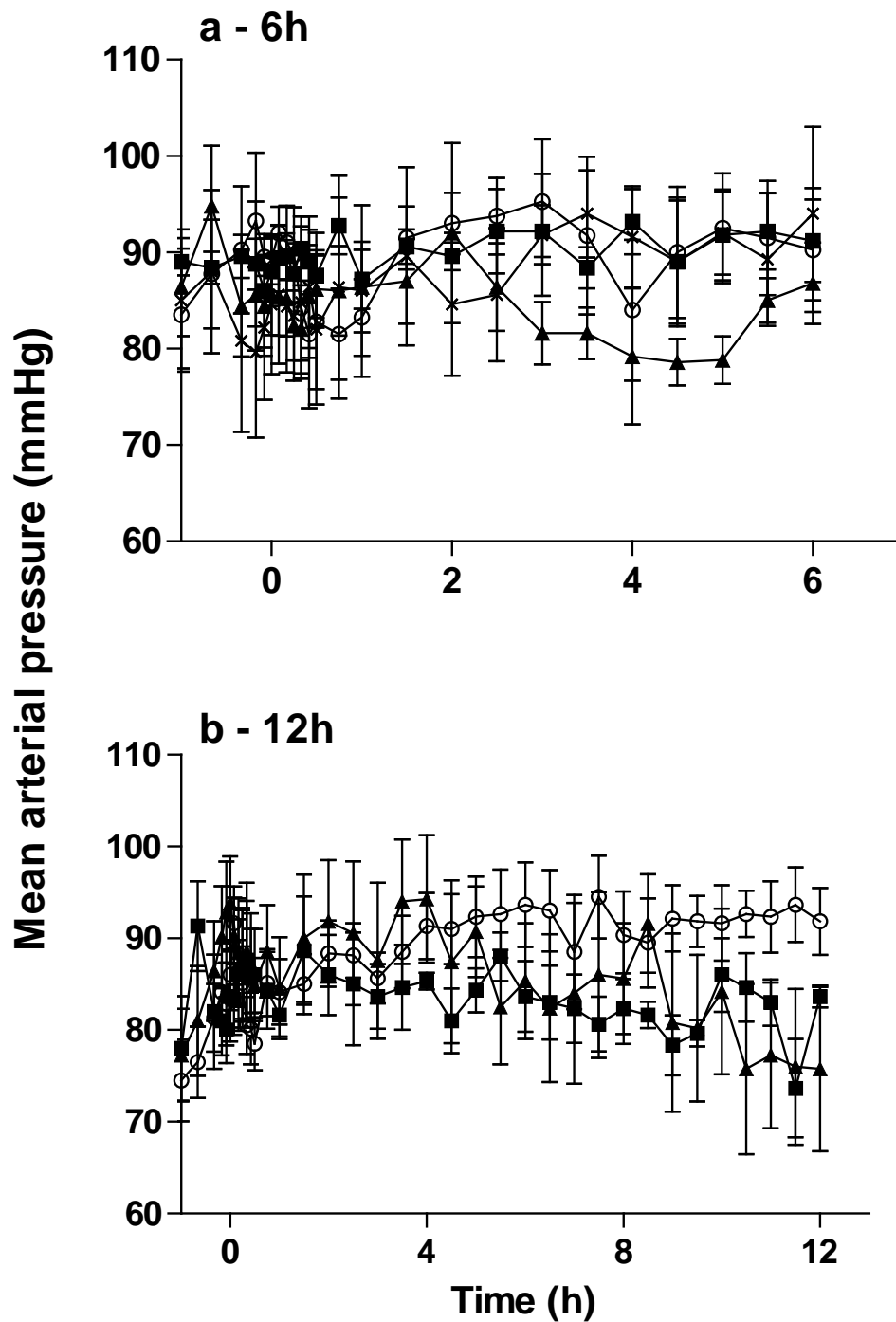


Changes in PVPI measured by thermodilution following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.

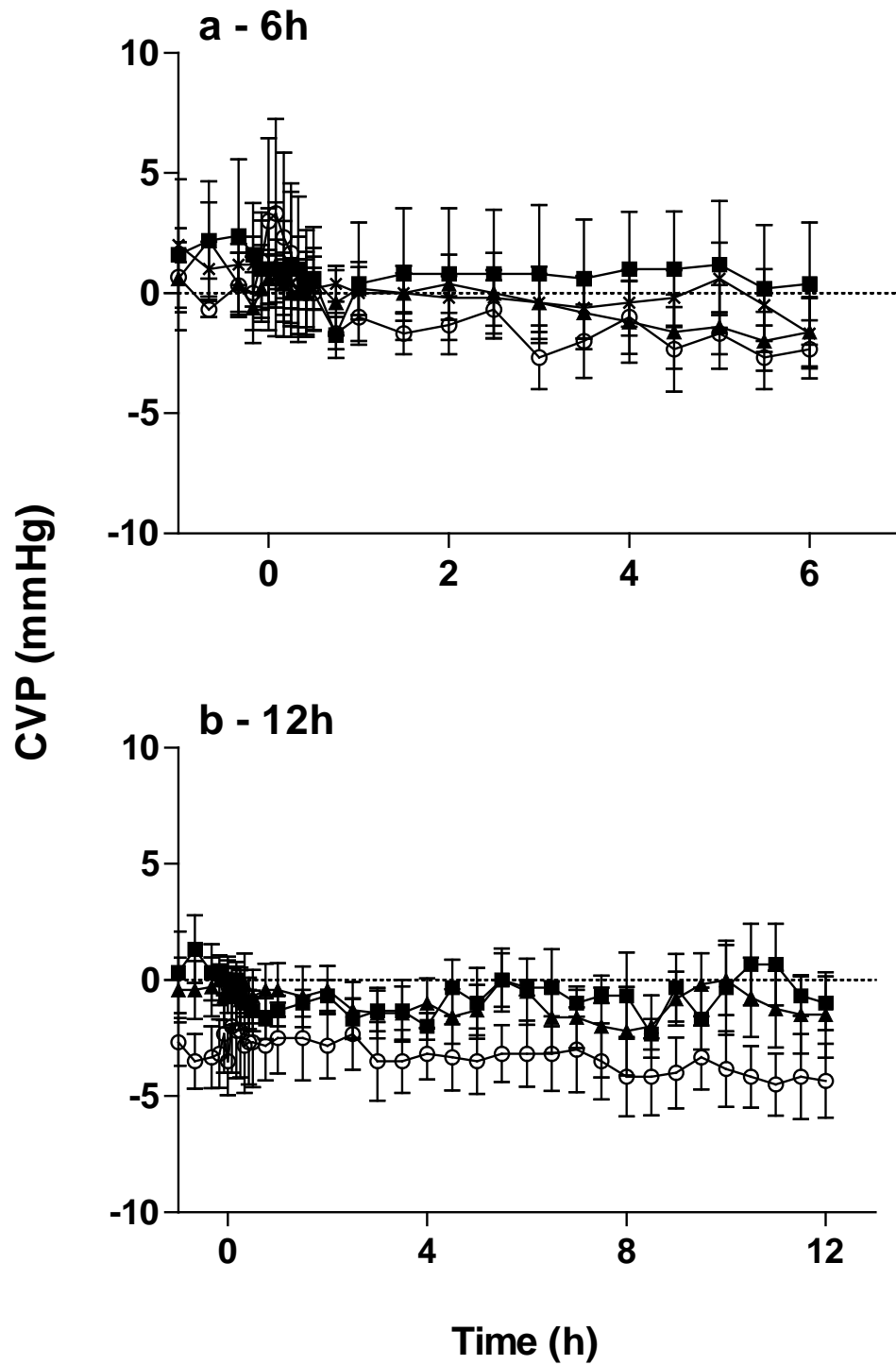
Parameters measured by Propaq



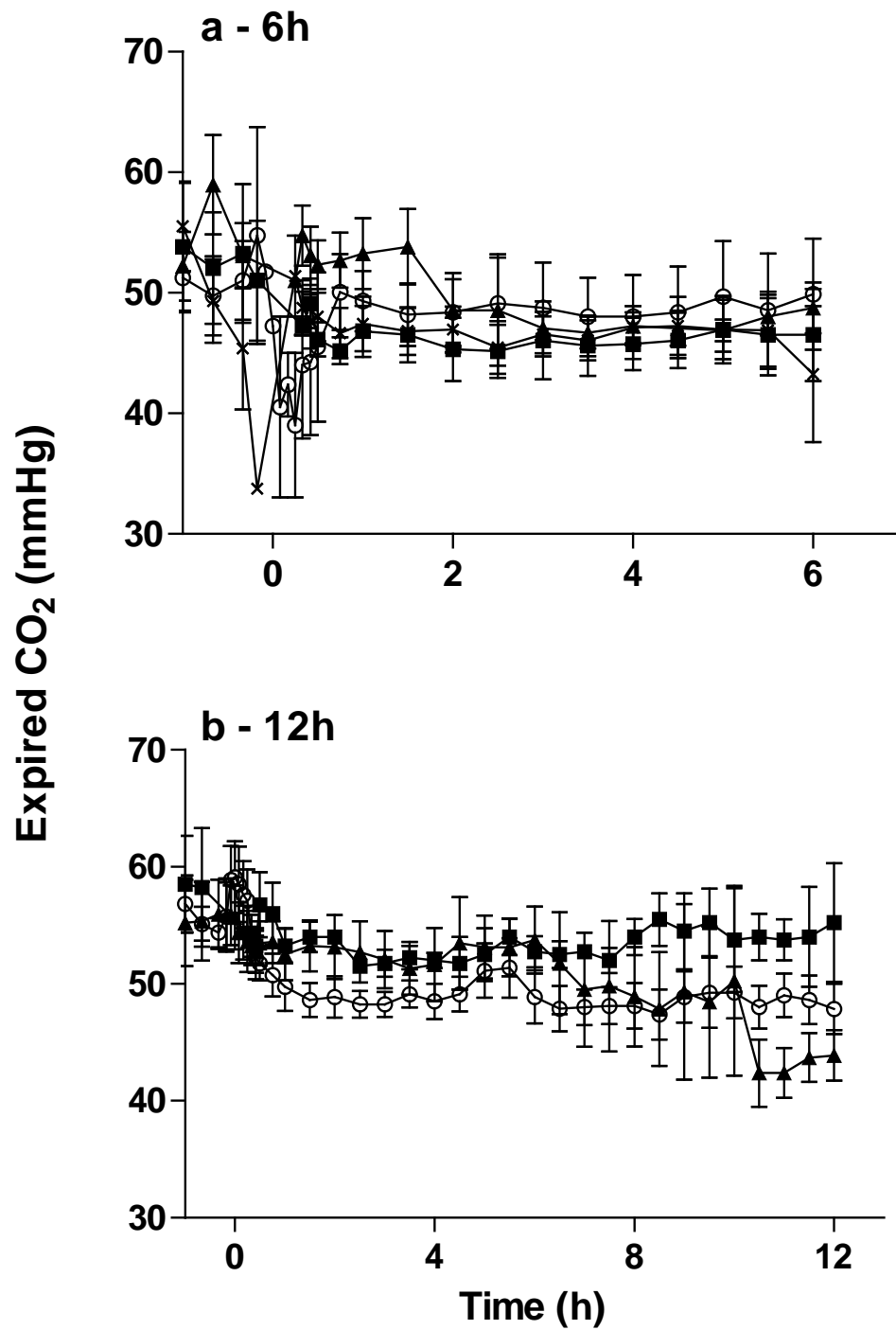
Changes in heart rate measured by propaq following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



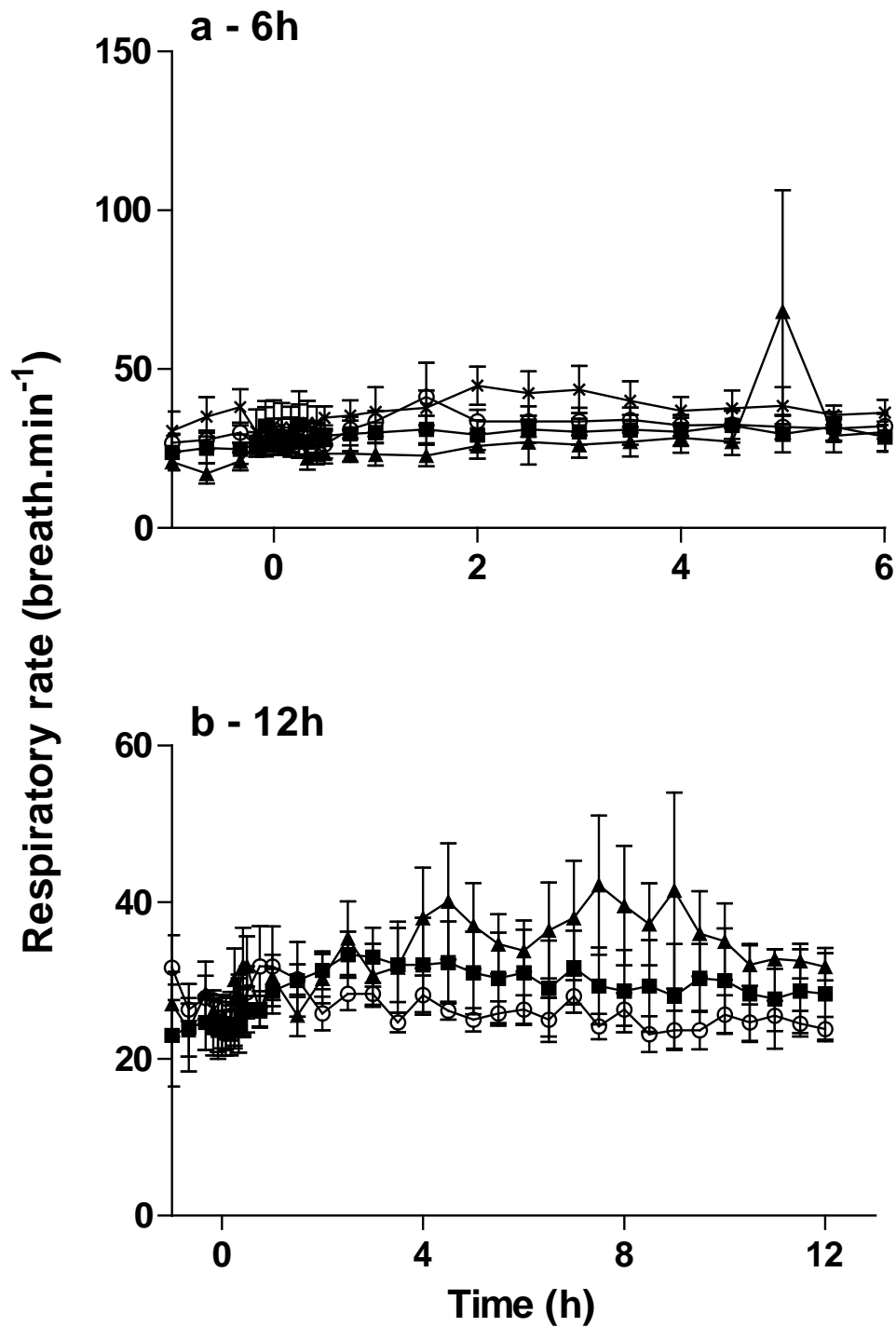
Changes in mean arterial pressure measured by propaq following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



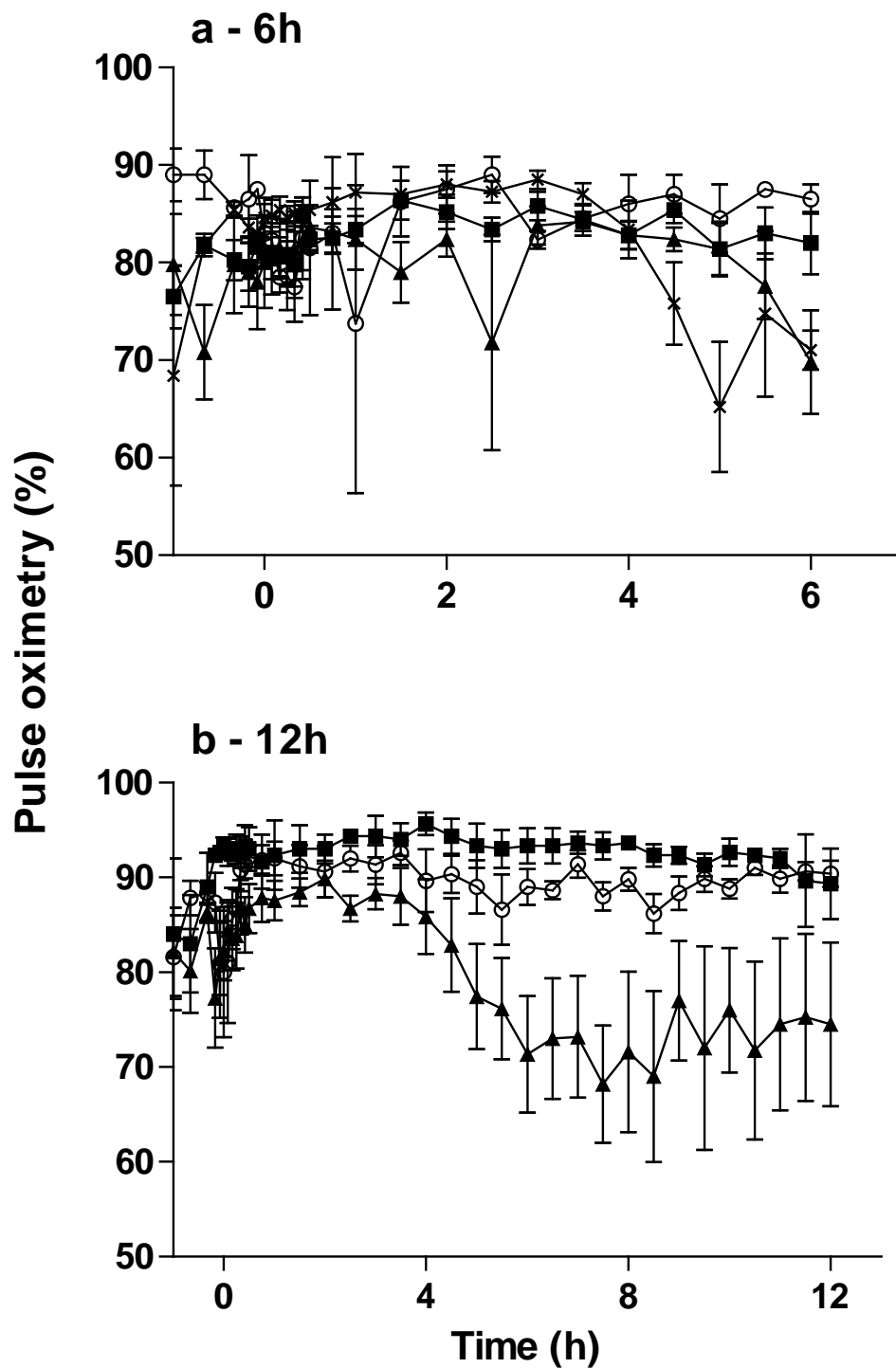
Changes in CVP measured by propaq following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (○). Data are expressed as mean \pm SE.



Changes in expired CO₂ measured by propaq following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.

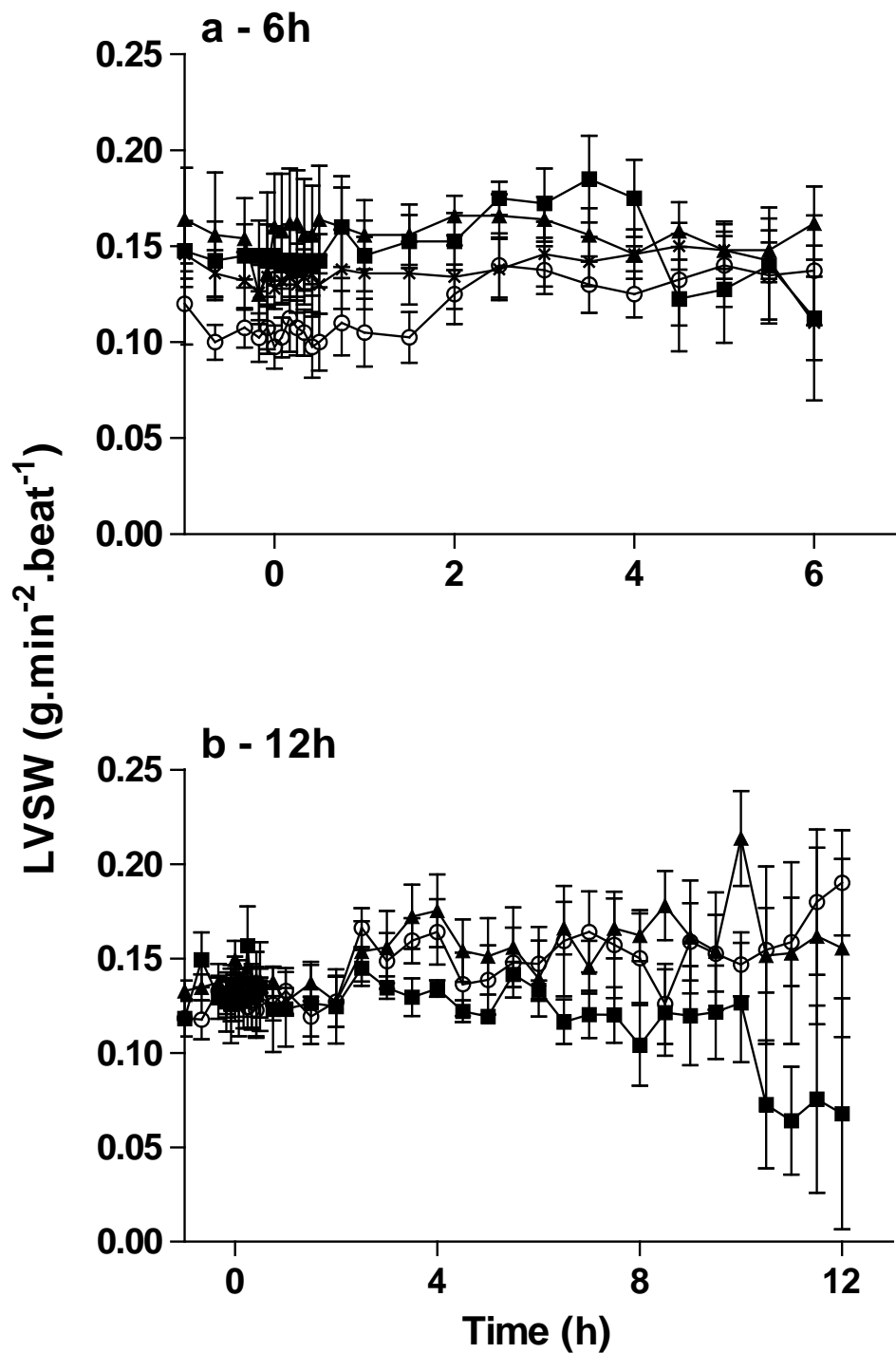


Changes in respiratory rate measured by propaq following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.

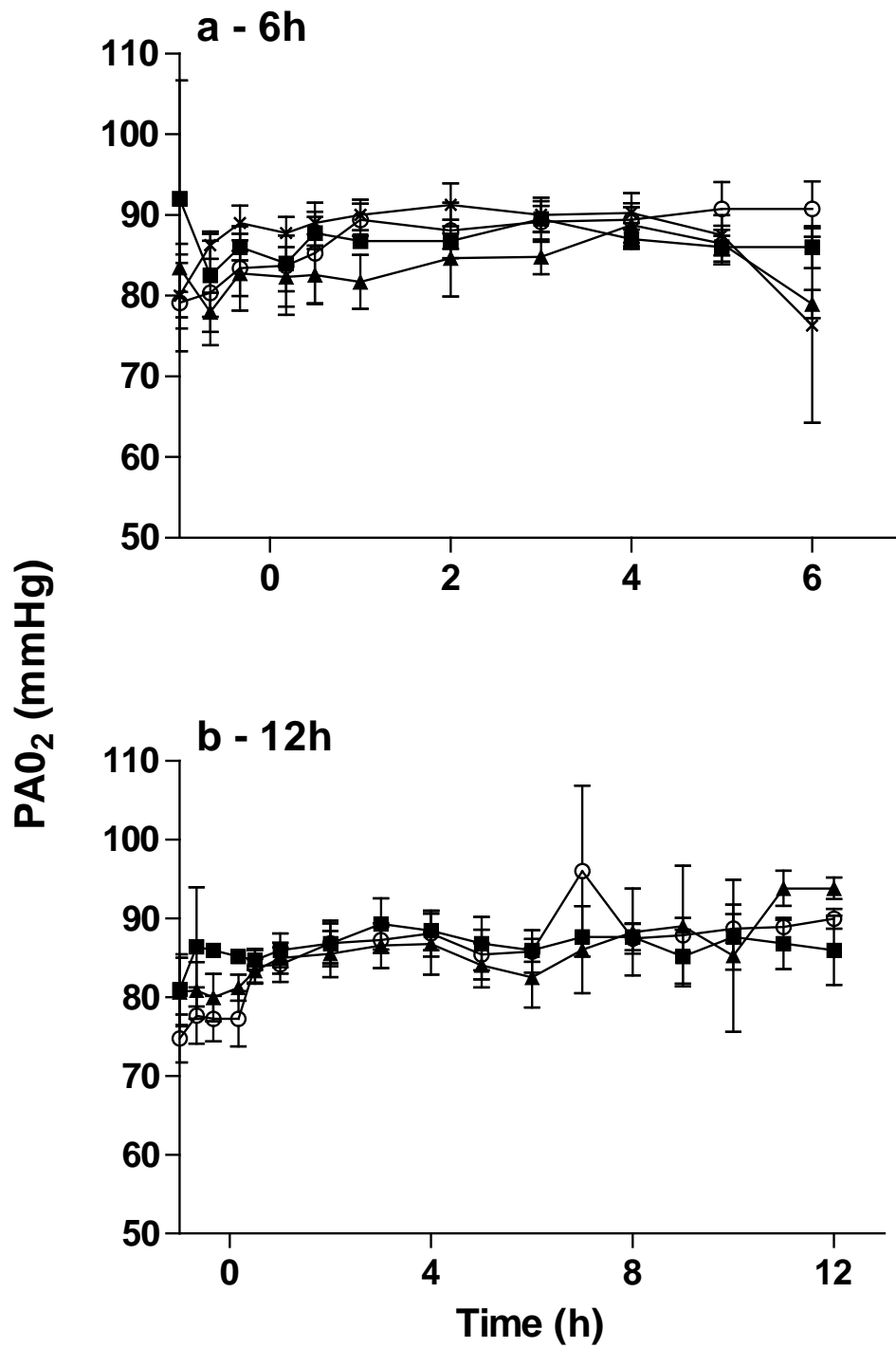


Changes in pulse oximetry measured by propaq following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.

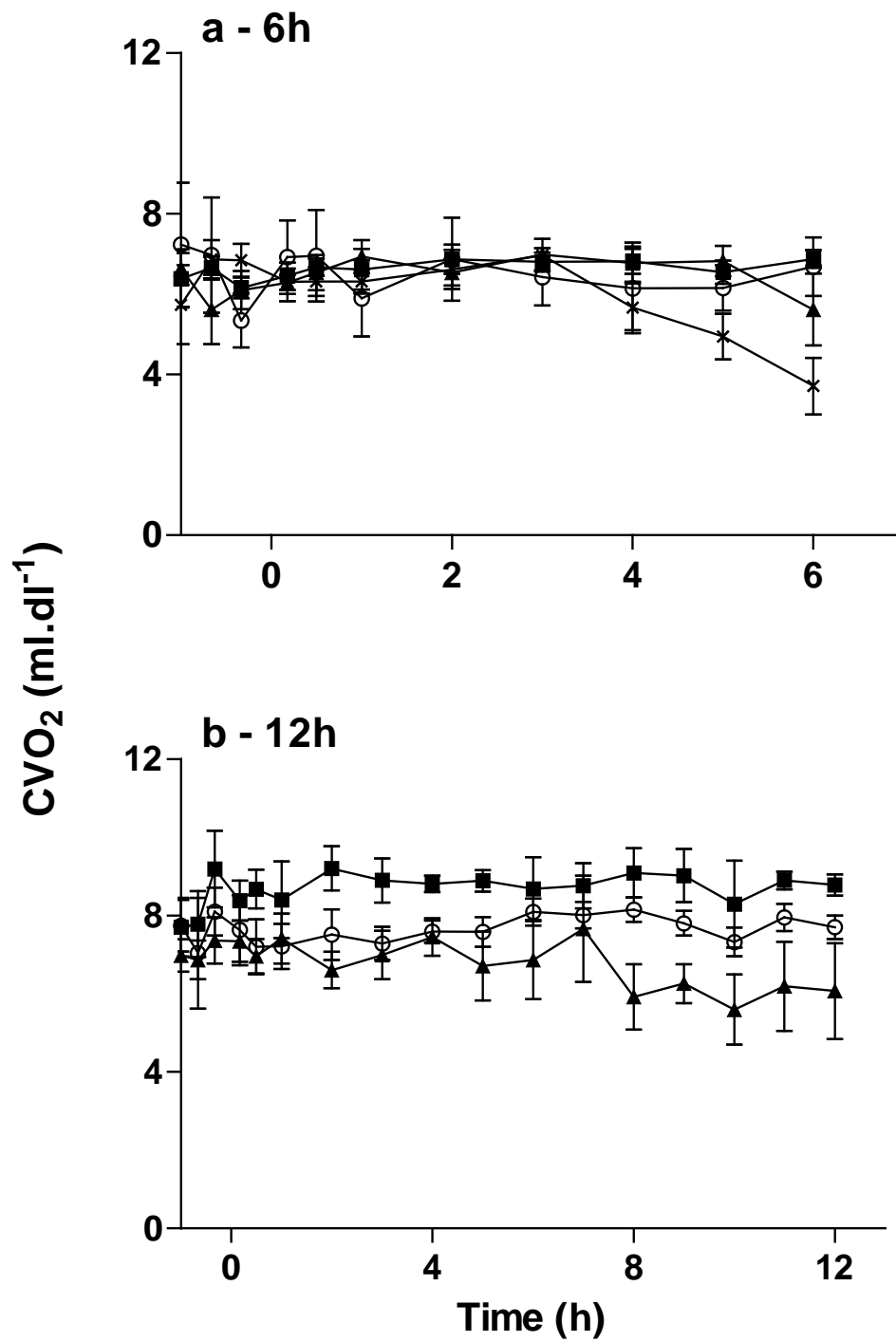
Derived Parameters



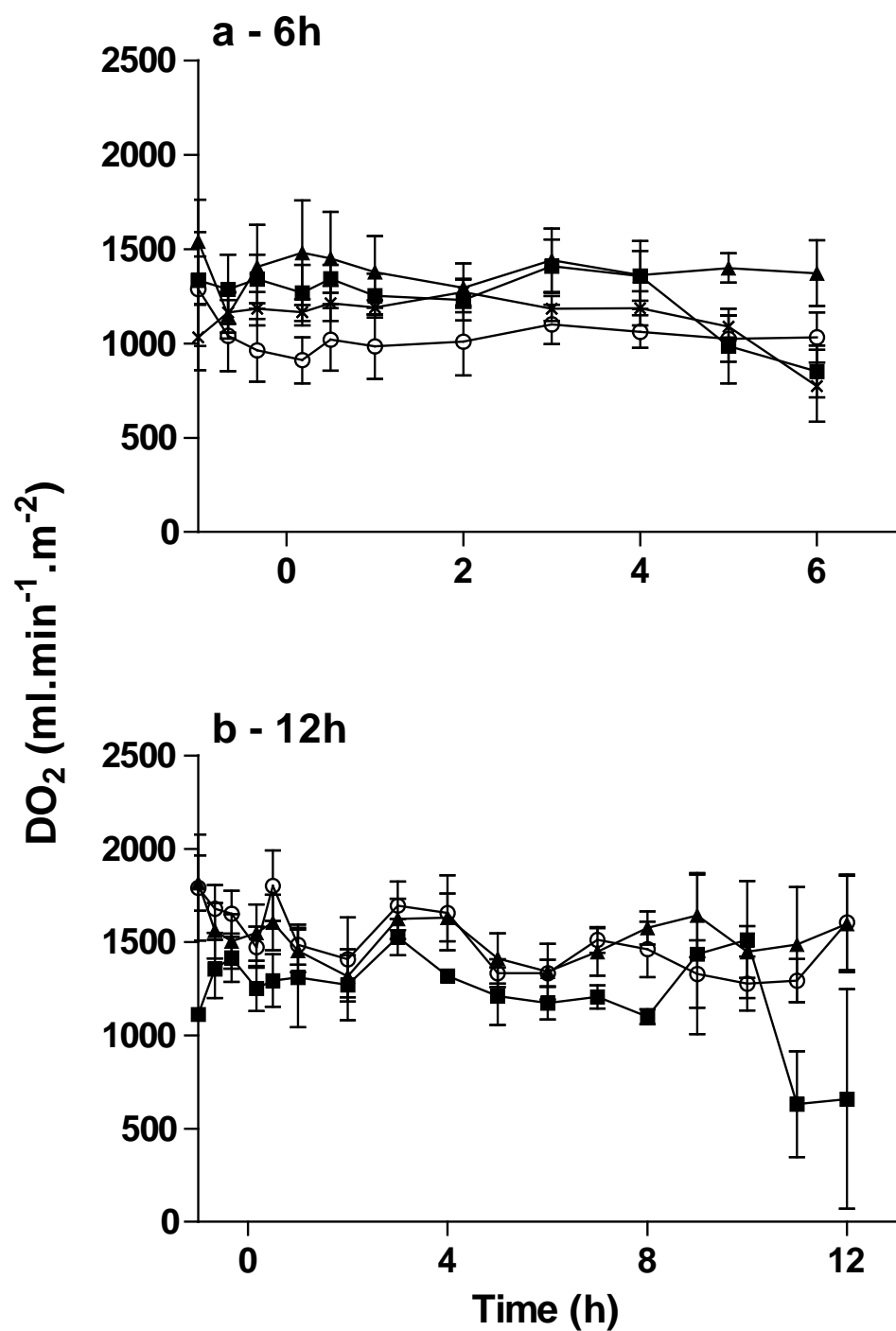
Changes in LVSW following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



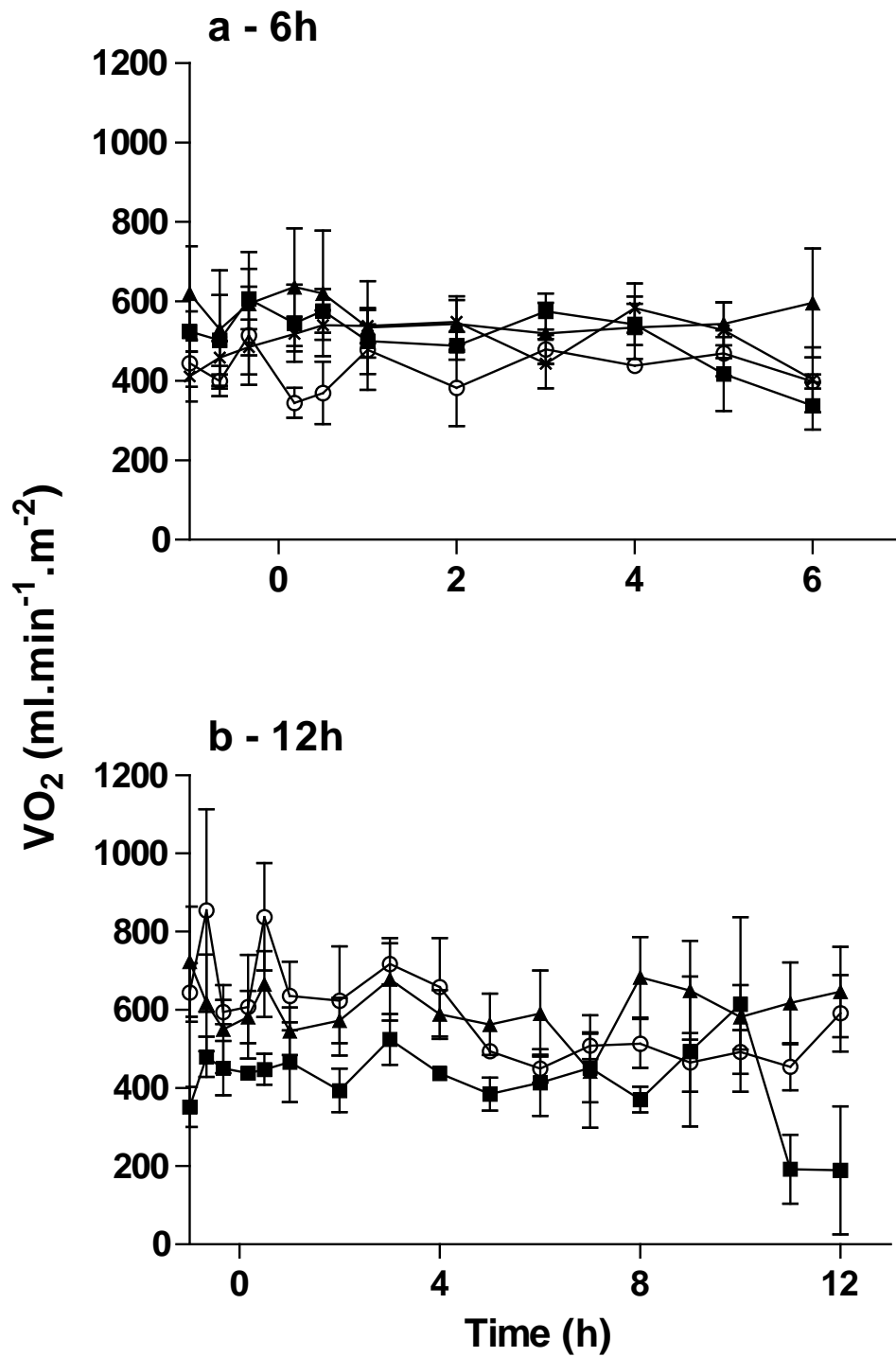
Changes in PAO₂ following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



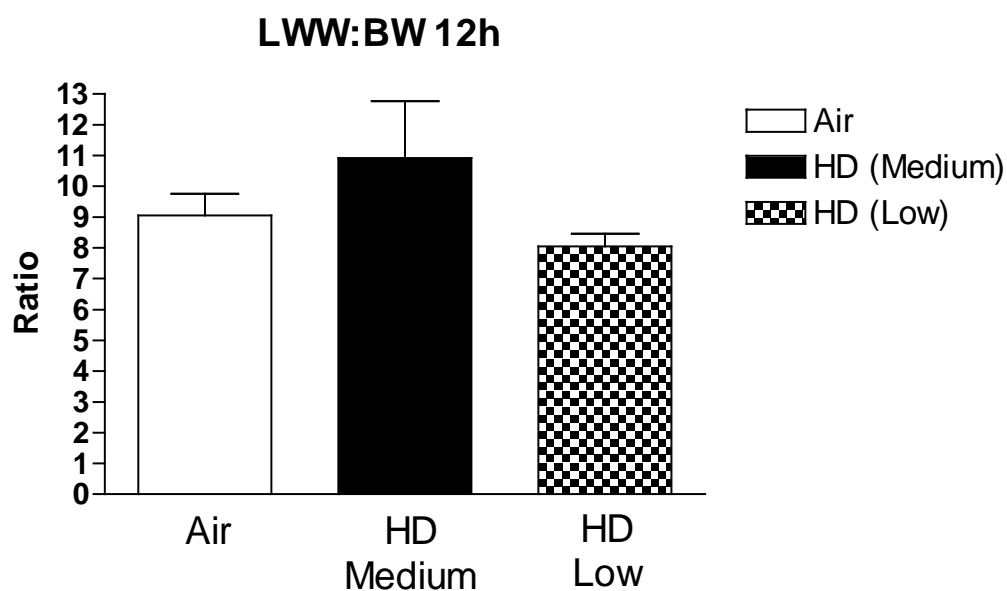
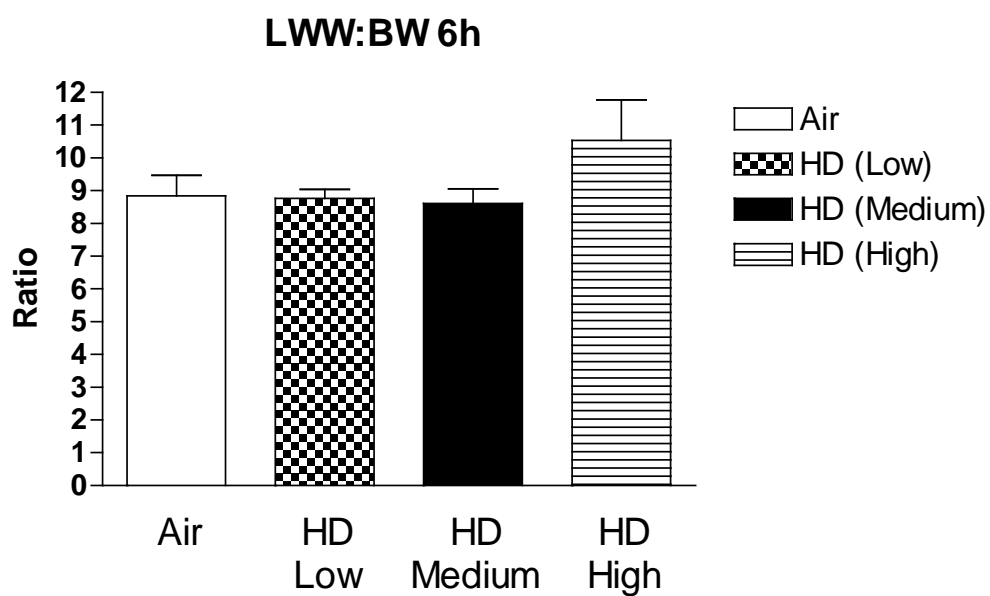
Changes in CvO₂ following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean ± SE.



Changes in DO₂ following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean ± SE.

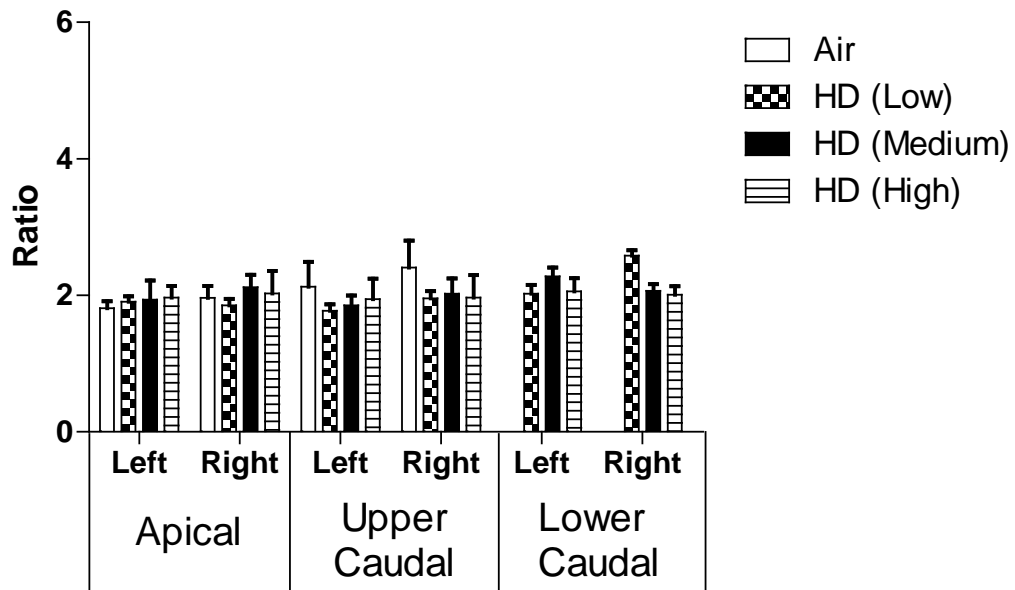


Changes in VO₂ following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean ± SE.

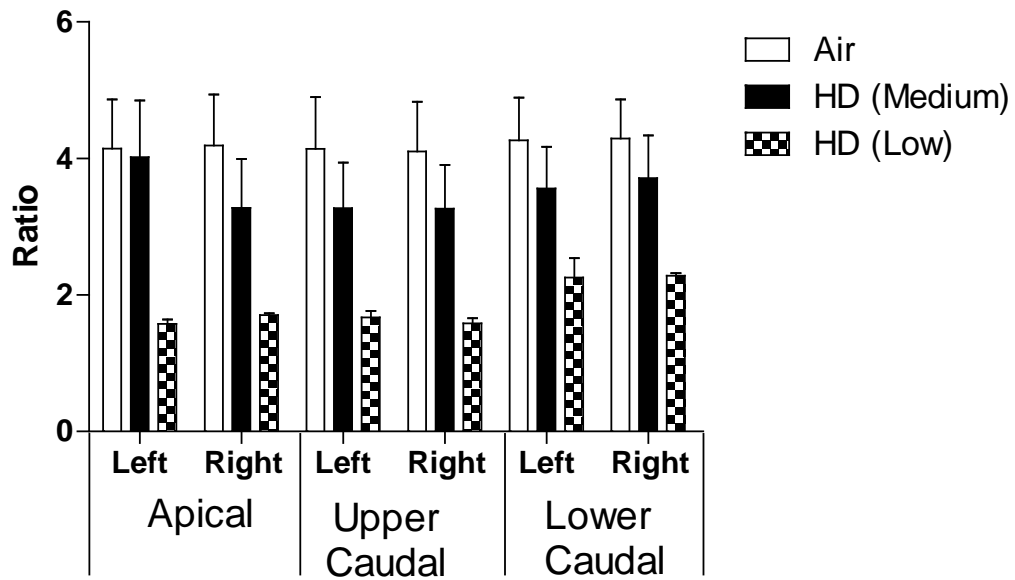


Changes in lung wet weight to body weight ratio following exposure to low, medium or high doses of HD, or exposure to air. Data are expressed as mean \pm SE.

LWW:DW 6h



LWW:DW 12h



Changes in lung wet weight to dry weight ratio following exposure to low, medium or high doses of HD, or exposure to air. Data are expressed as mean \pm SE.

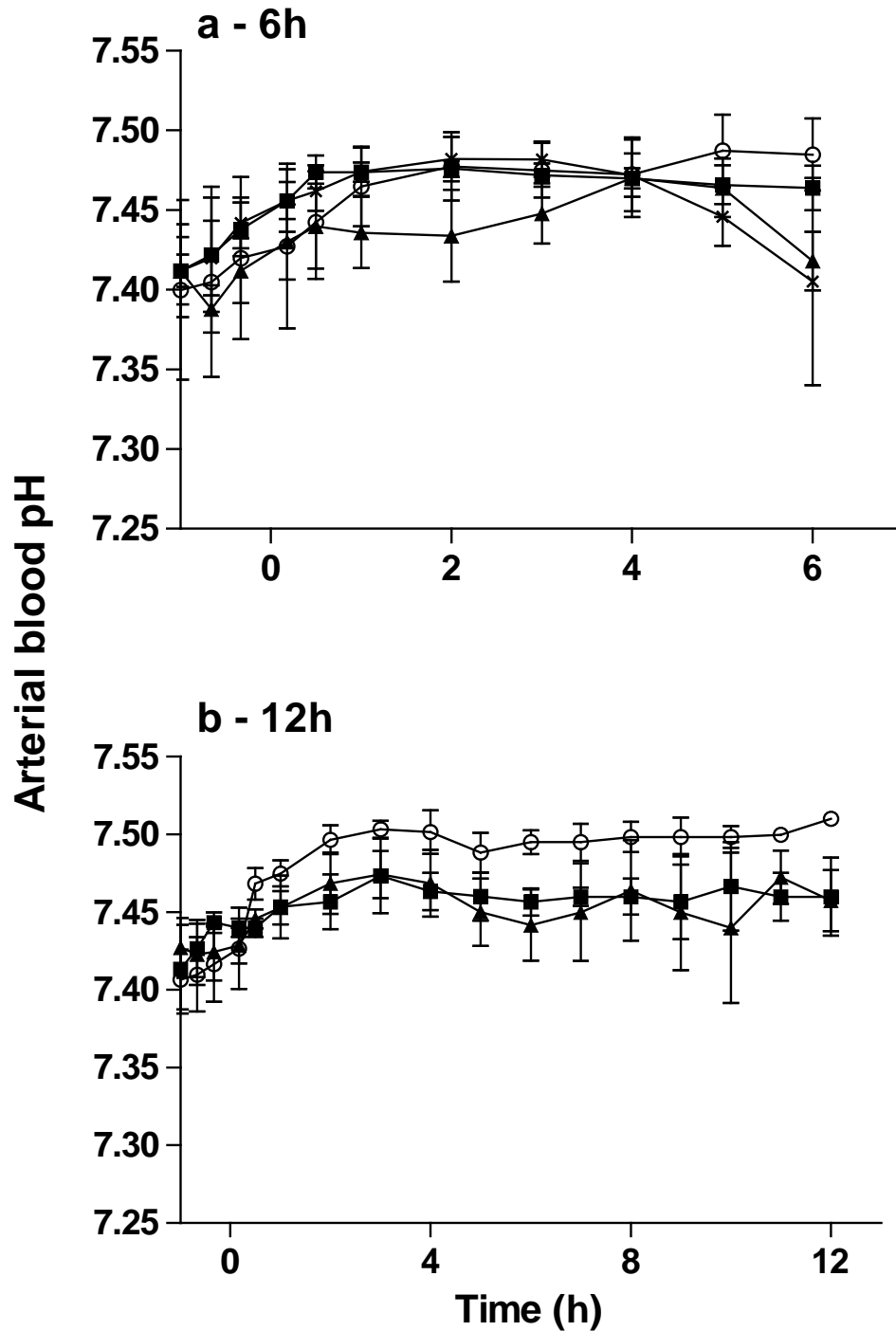
Blood and Cell Parameters

ALB	– Albumin
ALP	– Alkaline Phosphatase
ALT	– Alanine Aminotransferase
AST	– Aspartate Aminotransferase
BE(B)	– Base excess
Ca ²⁺	– Calcium concentration
Ca ²⁺ (7.4)	– Calcium concentration corrected for pH
COHb	– Carboxyhemoglobin
CK	– Creatinine kinase
CRP	– C Reactive Protein
GGT	– Gamma Glutamyl Transferase
HCO ₃ ⁻	– Bicarbonate concentration
HCO ₃ std	– Standard bicarbonate
Hct	– Hematocrit
Hgb	– Hemoglobin
HHb	– reduced Hemoglobin
IL-1β	– Interleukin-1β
IL-6	– Interleukin-6
IL-8	– Interleukin-8
MetHb	– Methemoglobin
mcv	– Mean cell volume
mch	– mean cell hemoglobin
mchc	– mean cell haemoglobin concentration
Na ⁺	– Sodium concentration
O ₂ ct	– Oxygen content
O ₂ Hb	– Oxyhemoglobin

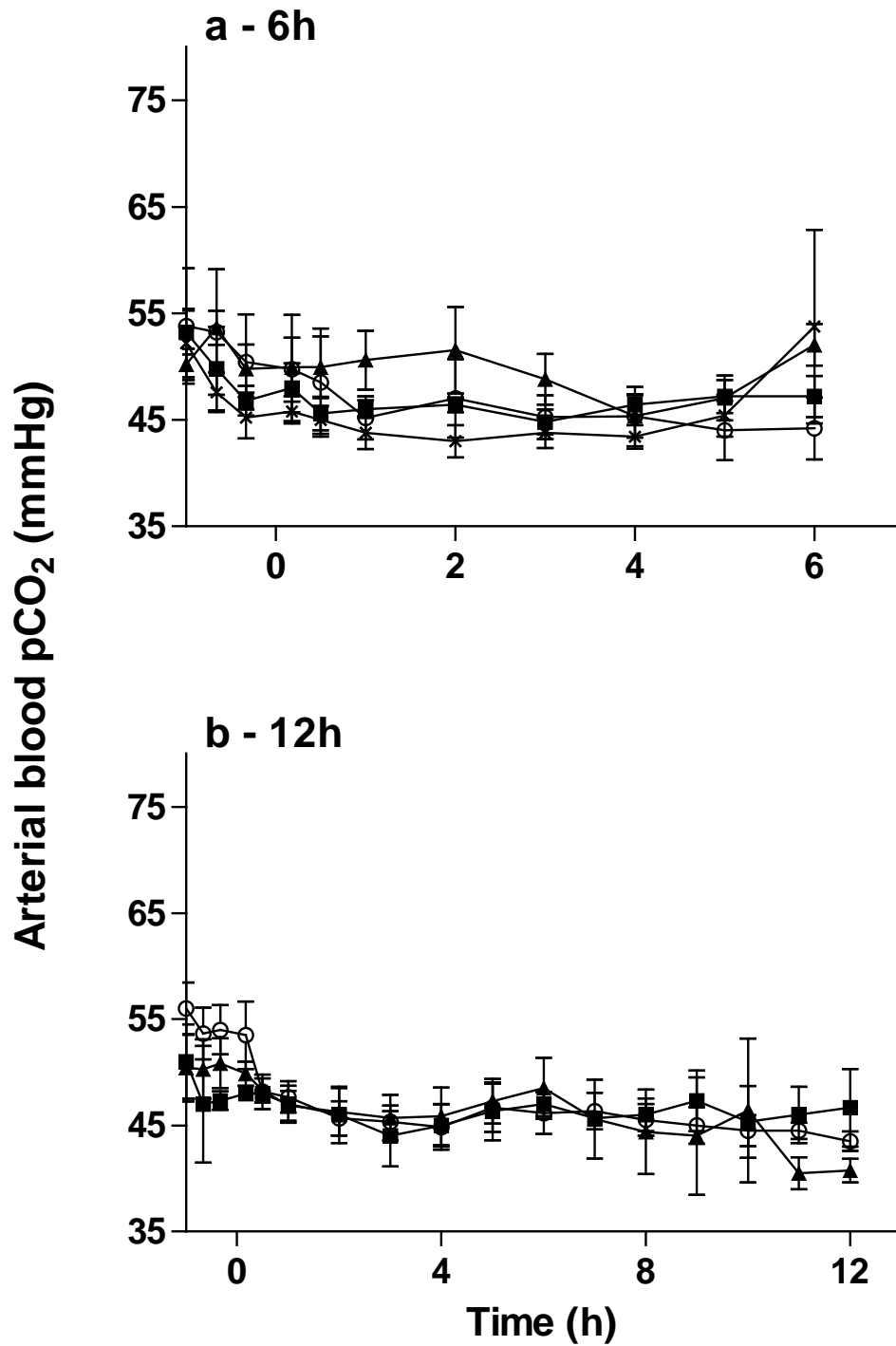
UNCLASSIFIED

pCO ₂	– Partial pressure of carbon dioxide in blood
pCO ₂ (t) temperature	– Partial pressure of carbon dioxide in blood corrected for temperature
pH	– Measure of acidity or basicity of a solution
pH(t) temperature	– Measure of acidity or basicity of a solution corrected for temperature
PLT	– Platelets
pO ₂	– Partial pressure of oxygen in blood
pO ₂ (t)	– Partial pressure of oxygen in blood corrected for temperature
RBC	– Red Blood Cells
RHb	– Reduced hemoglobin
SO ₂	– Oxygen saturation
TCO ₂	– Total carbon dioxide
THb	– Total Hemoglobin
TNF- α	– Tumour necrosis factor alpha
WBC	– White Blood Cells
WBC(diffs)	– White blood cell differentials (identification of individual cells e.g. neutrophils, eosinophils, lymphocytes, Alveolar macrophages etc)

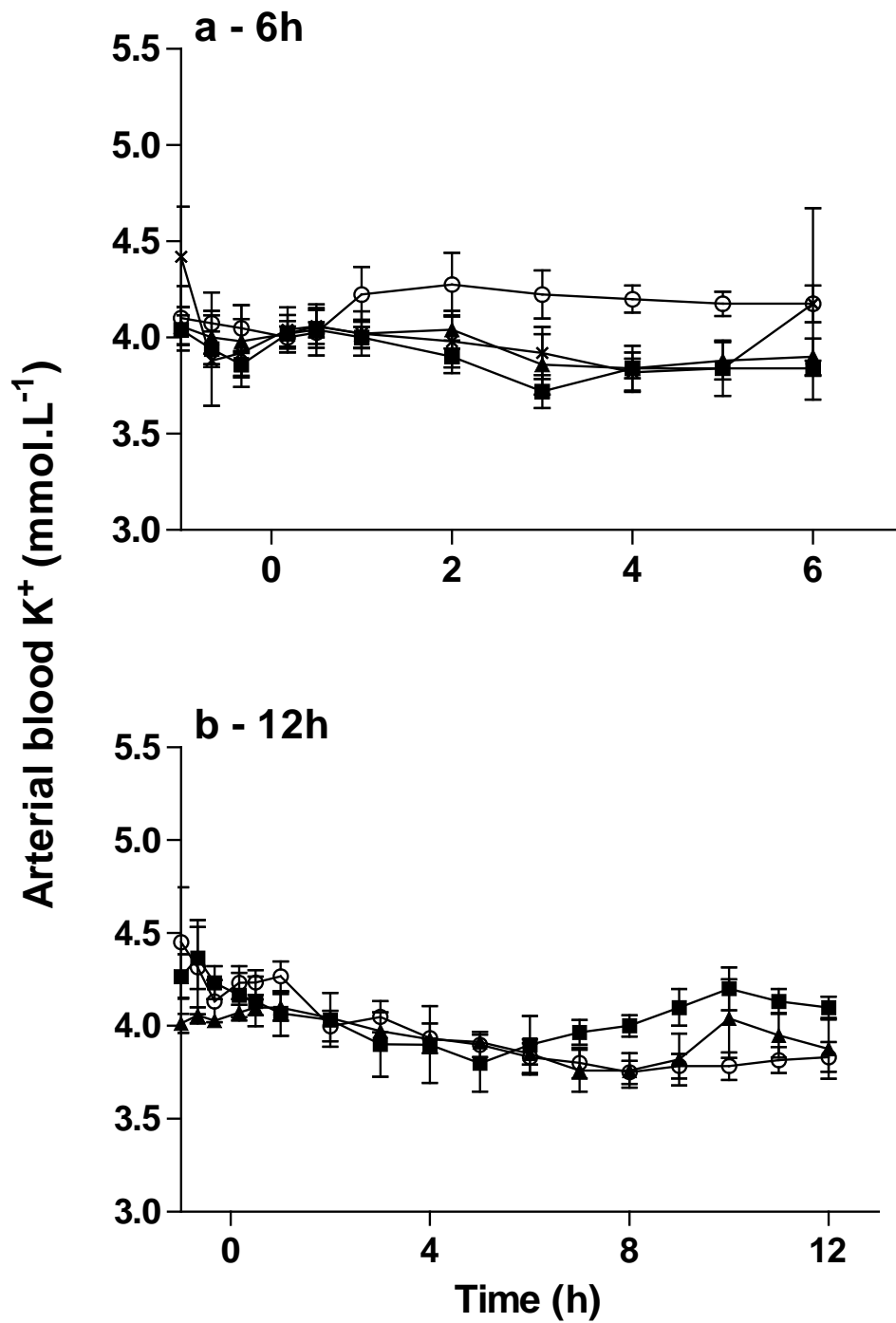
Blood Gas Parameters – Arterial



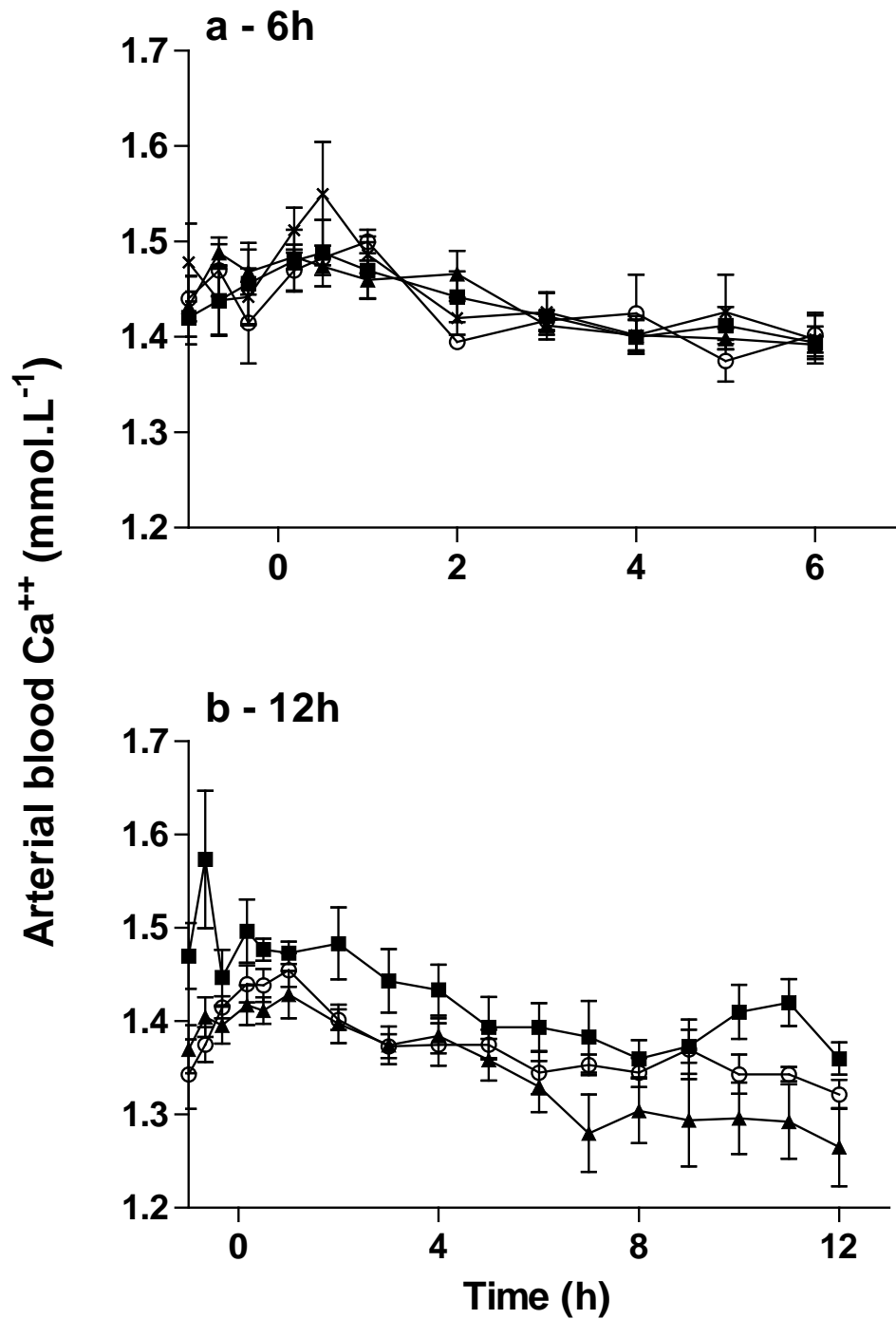
Changes in arterial blood pH following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (○). Data are expressed as mean \pm SE.



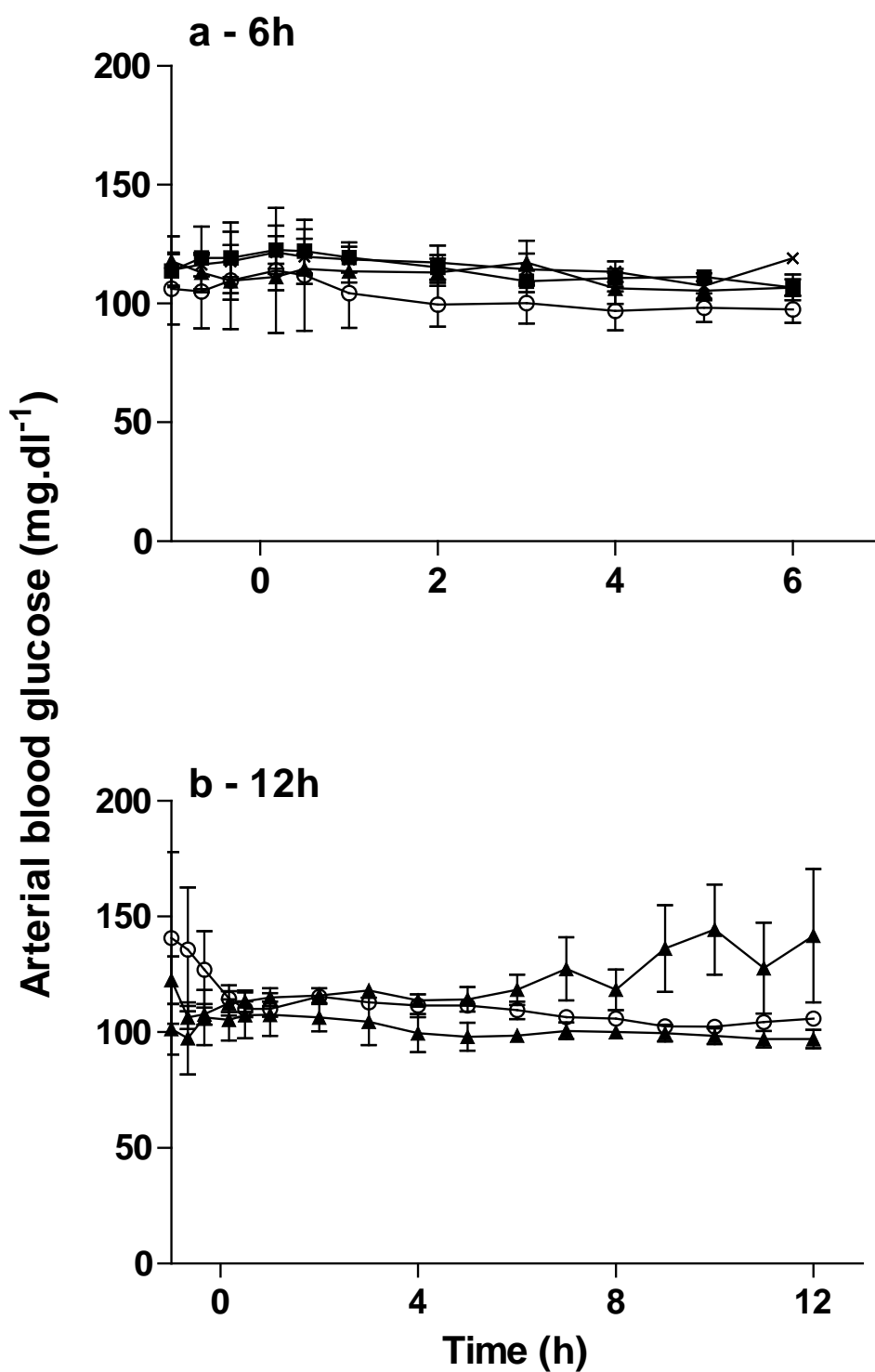
Changes in arterial blood pCO₂ following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean ± SE.



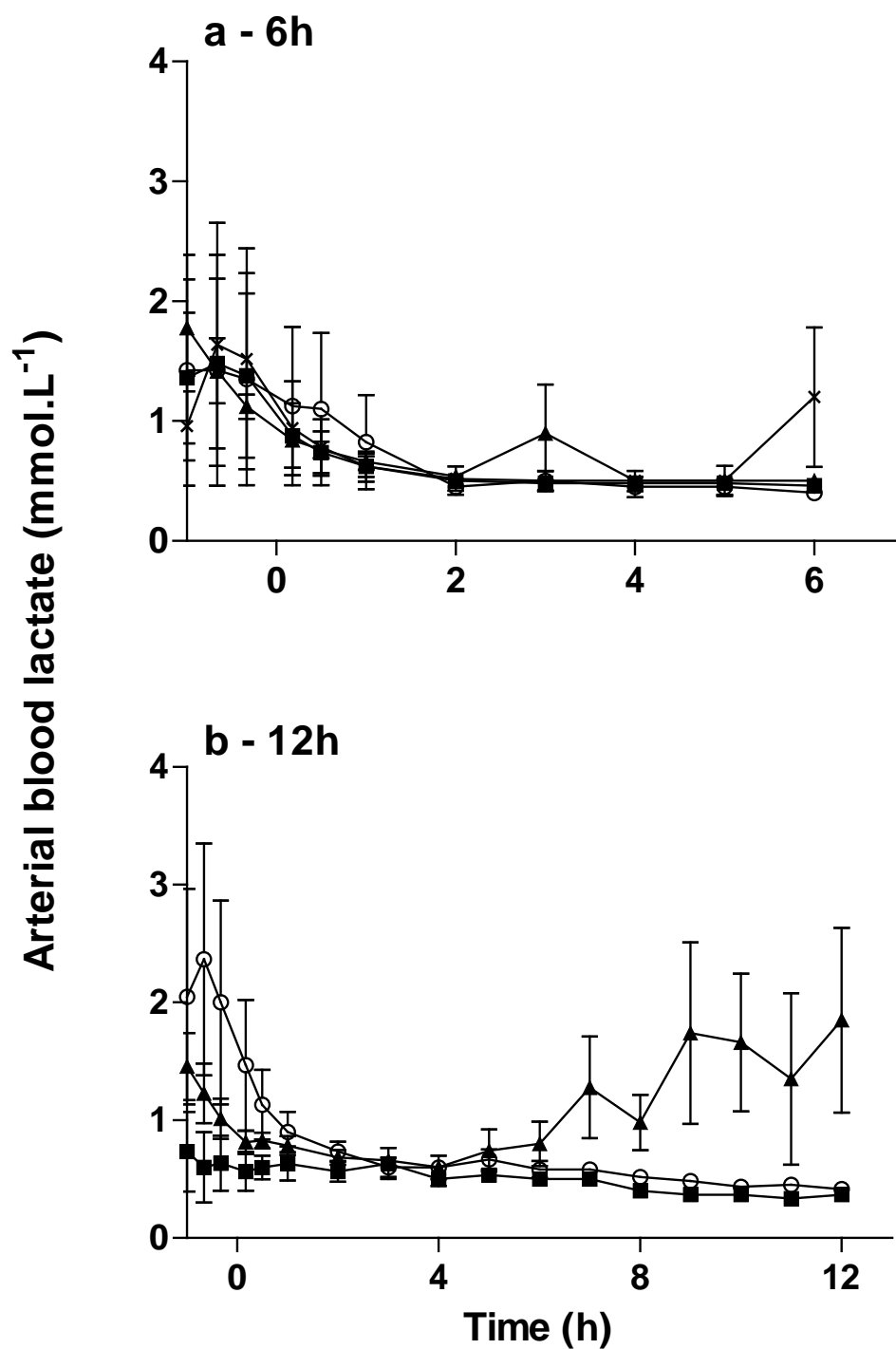
Changes in arterial blood potassium following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (○). Data are expressed as mean \pm SE.



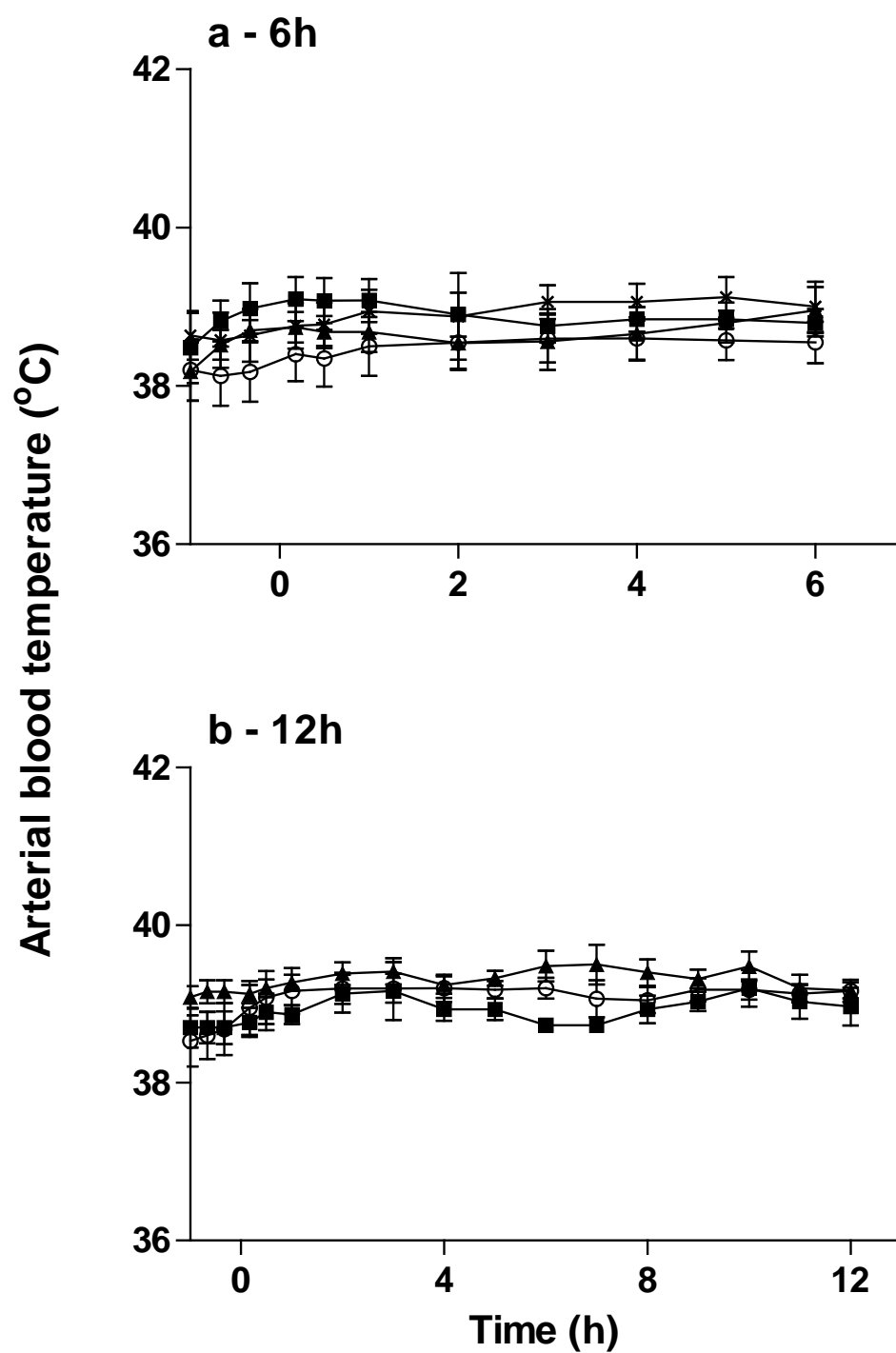
Changes in arterial blood calcium following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



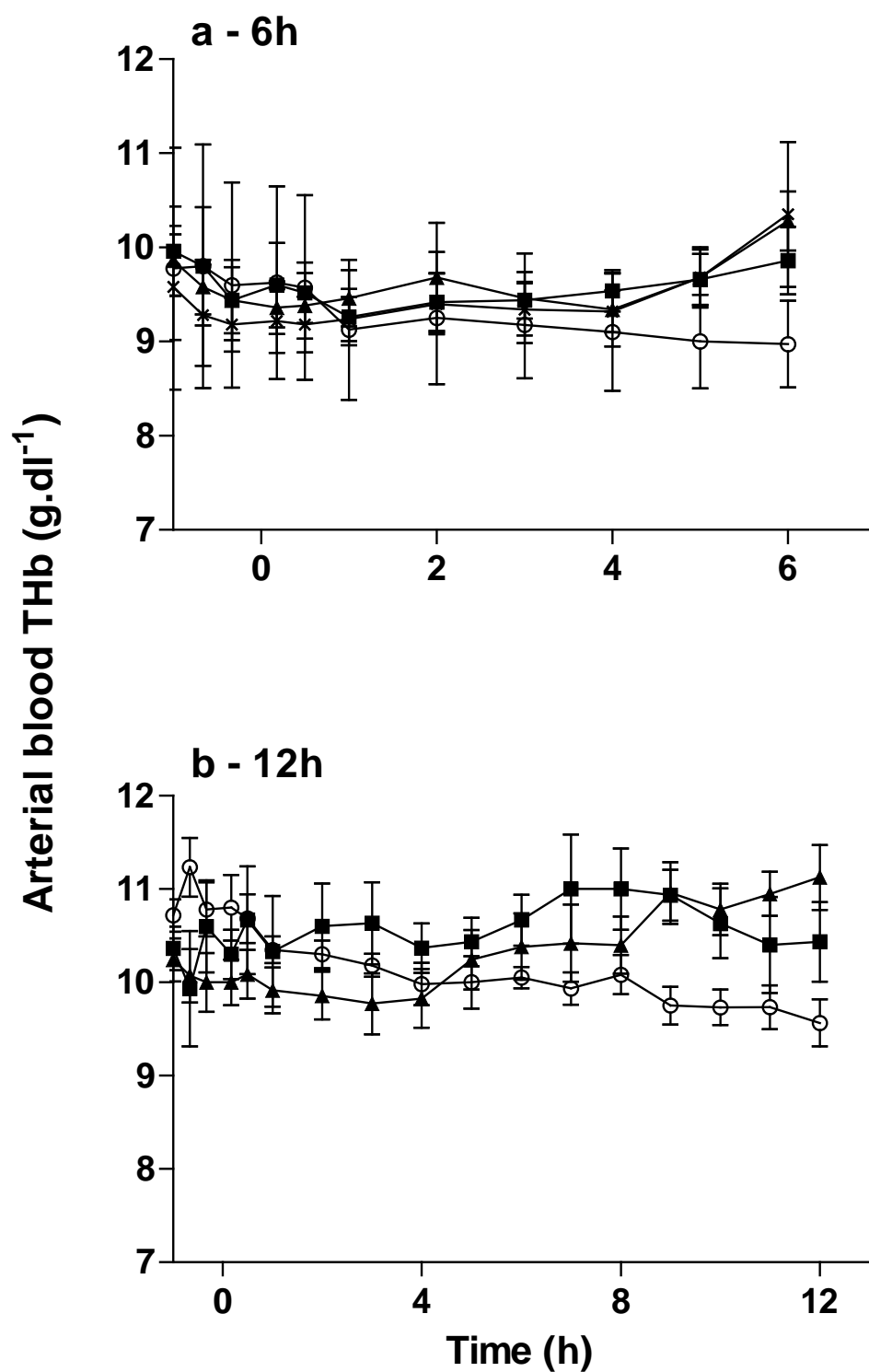
Changes in arterial blood glucose following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (○). Data are expressed as mean \pm SE.



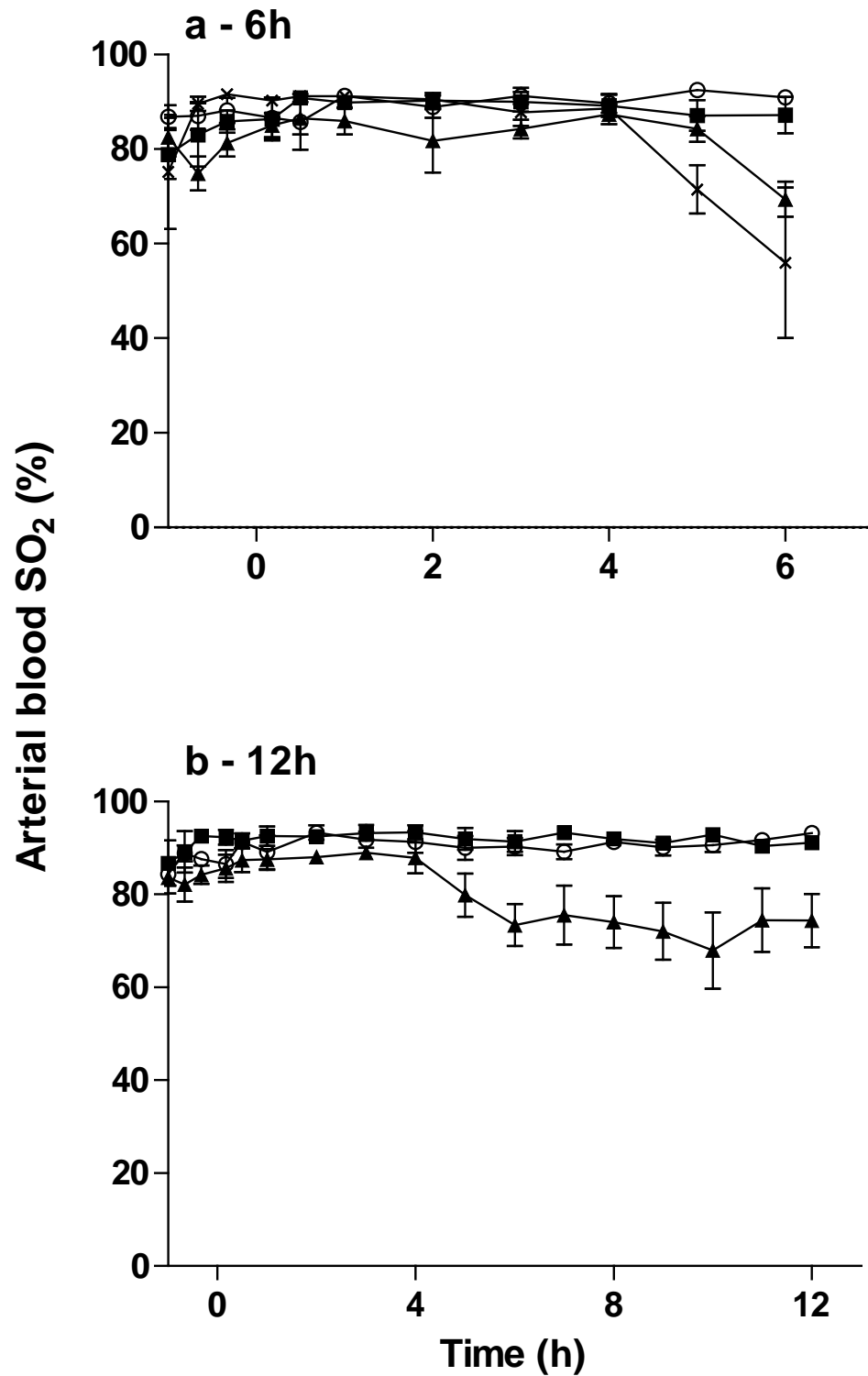
Changes in arterial blood lactate following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



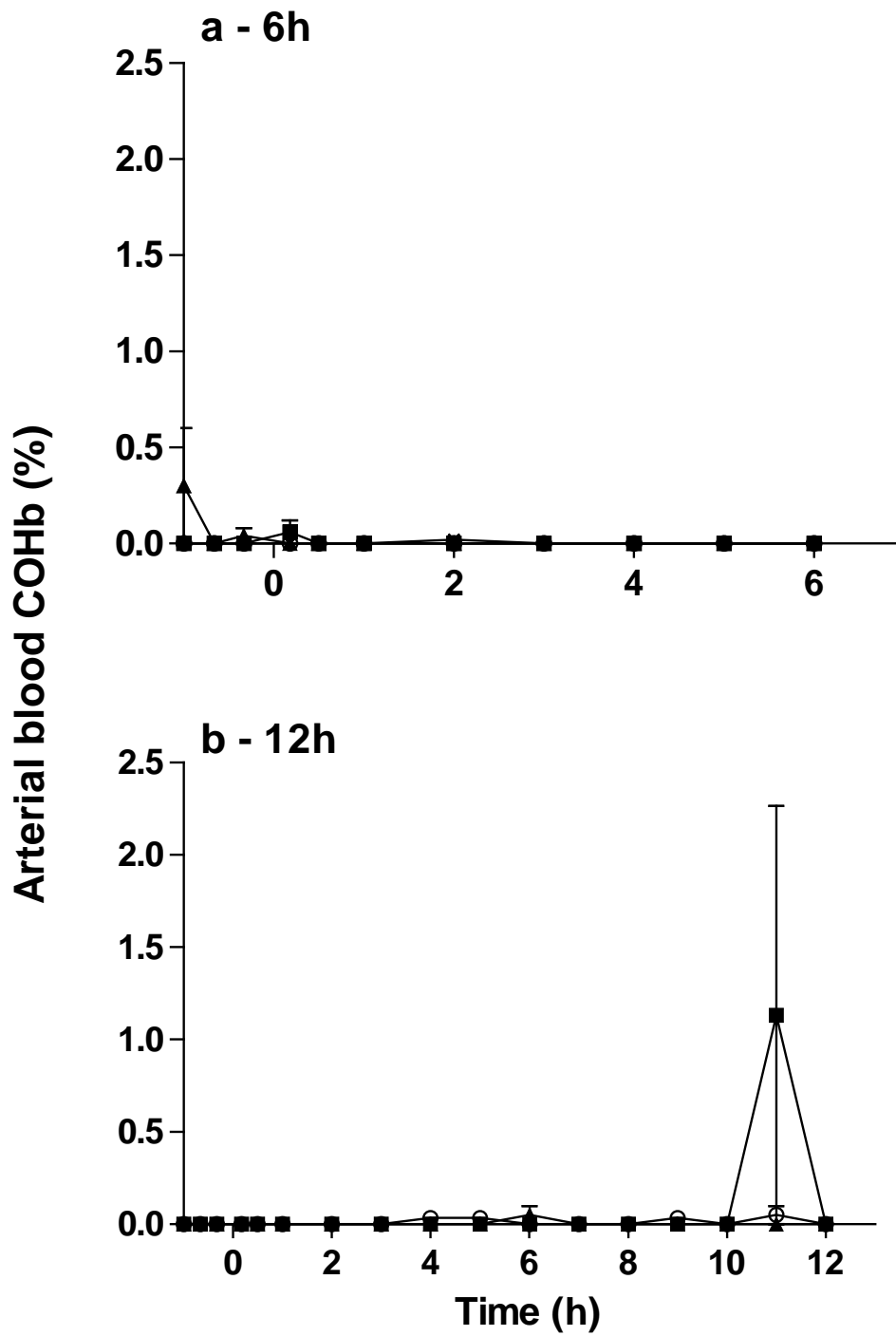
Changes in arterial blood temperature following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



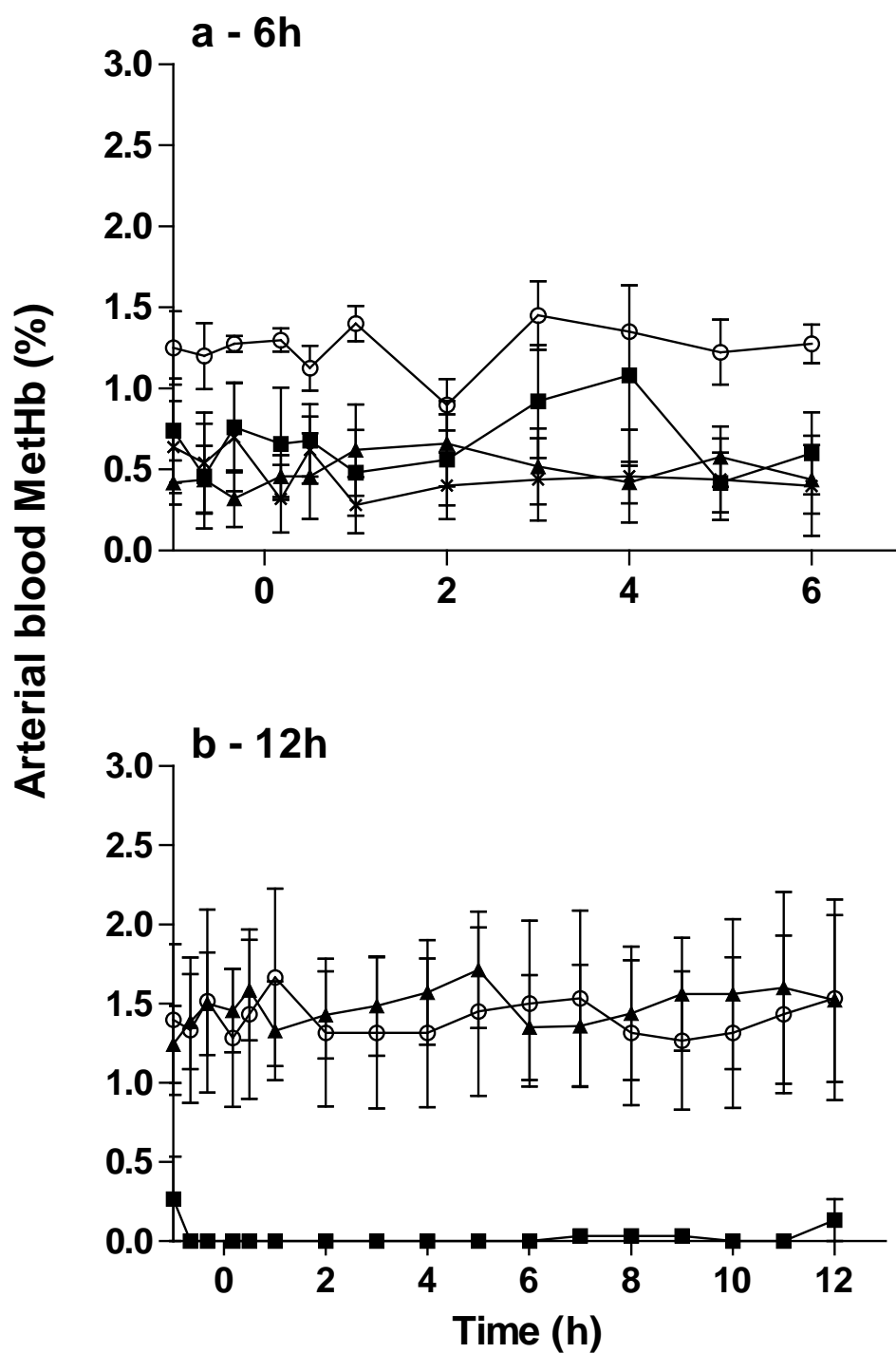
Changes in arterial blood THb following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



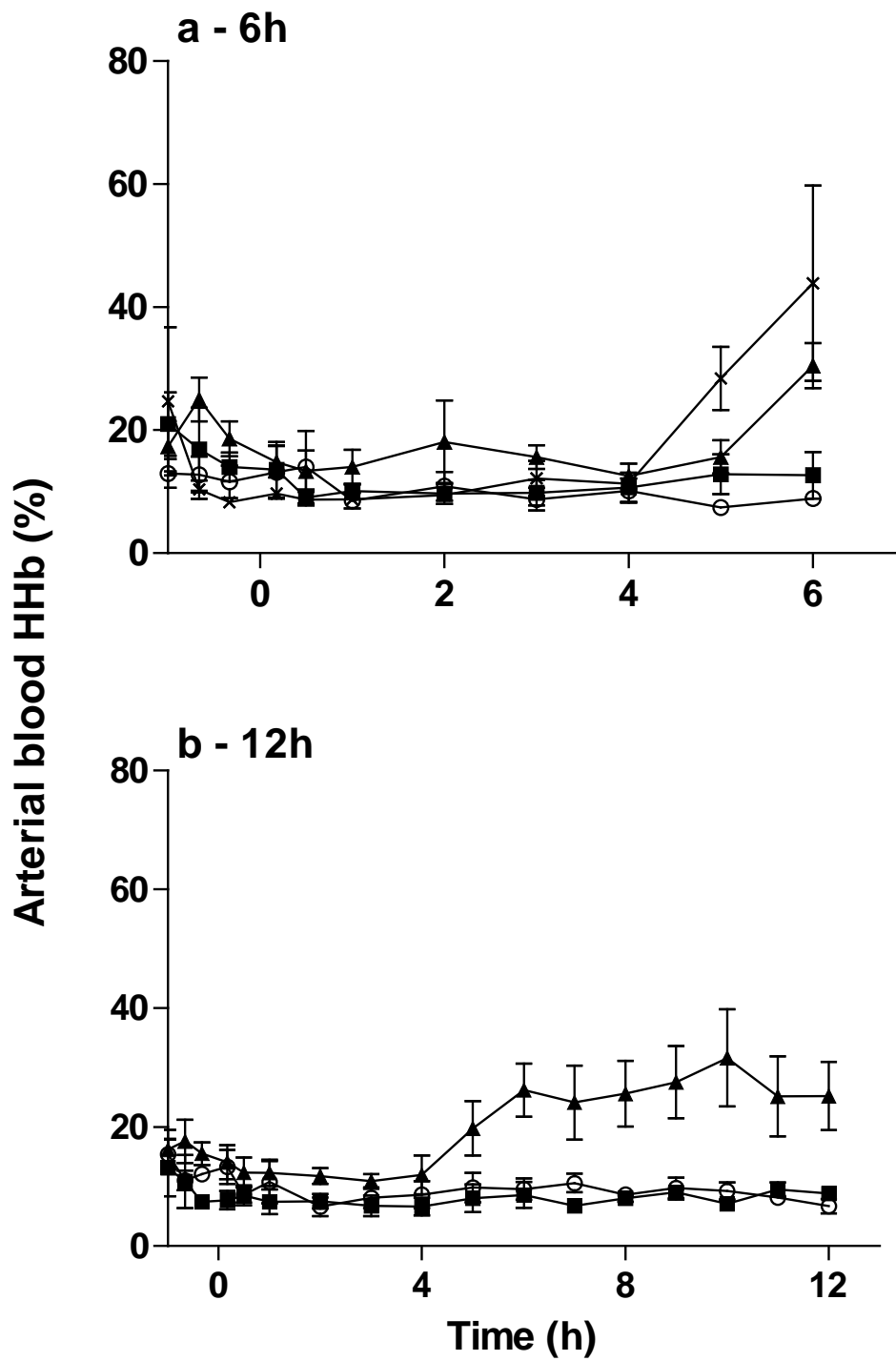
Changes in arterial blood SO_2 following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



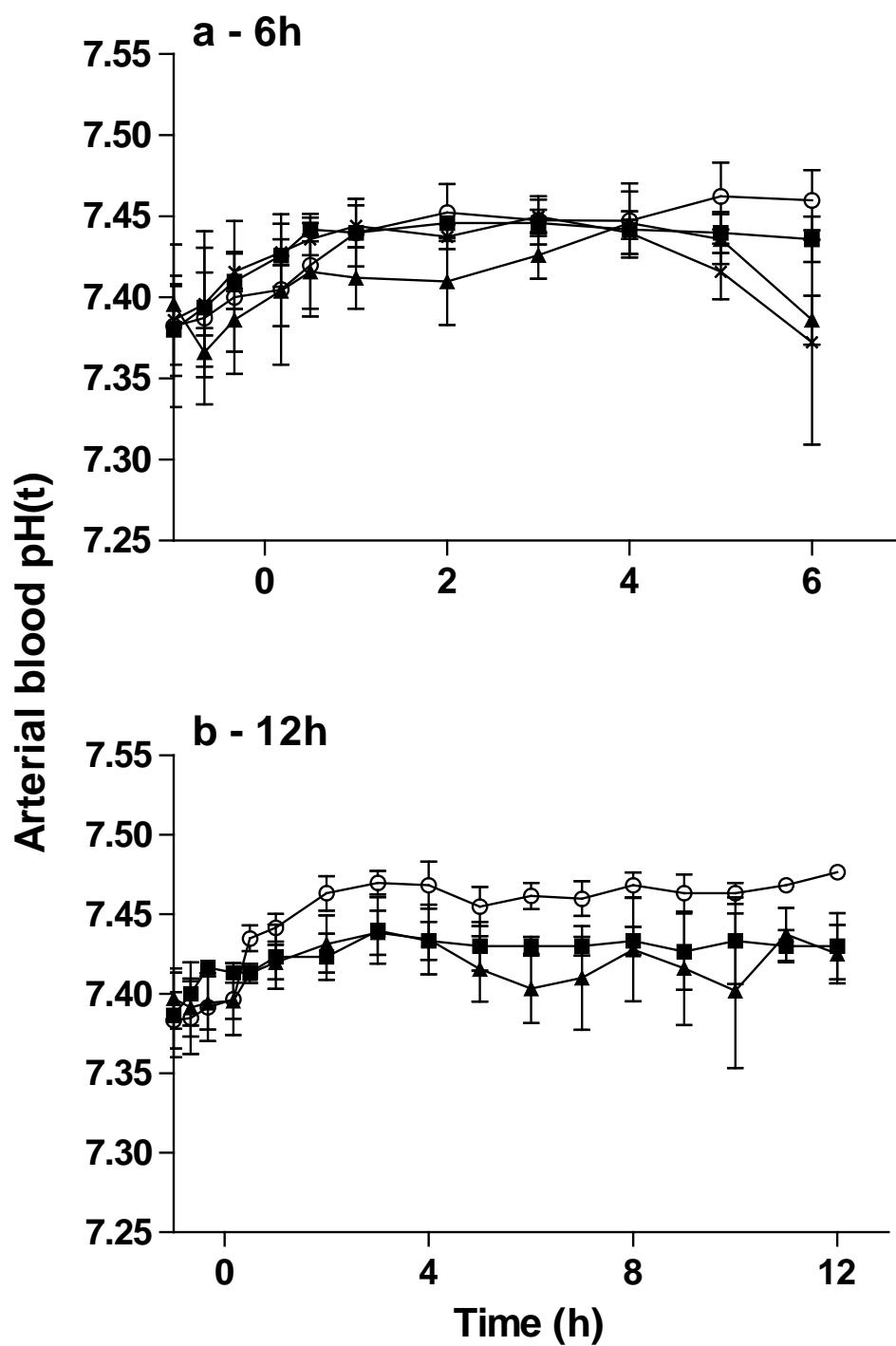
Changes in arterial blood COHb following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



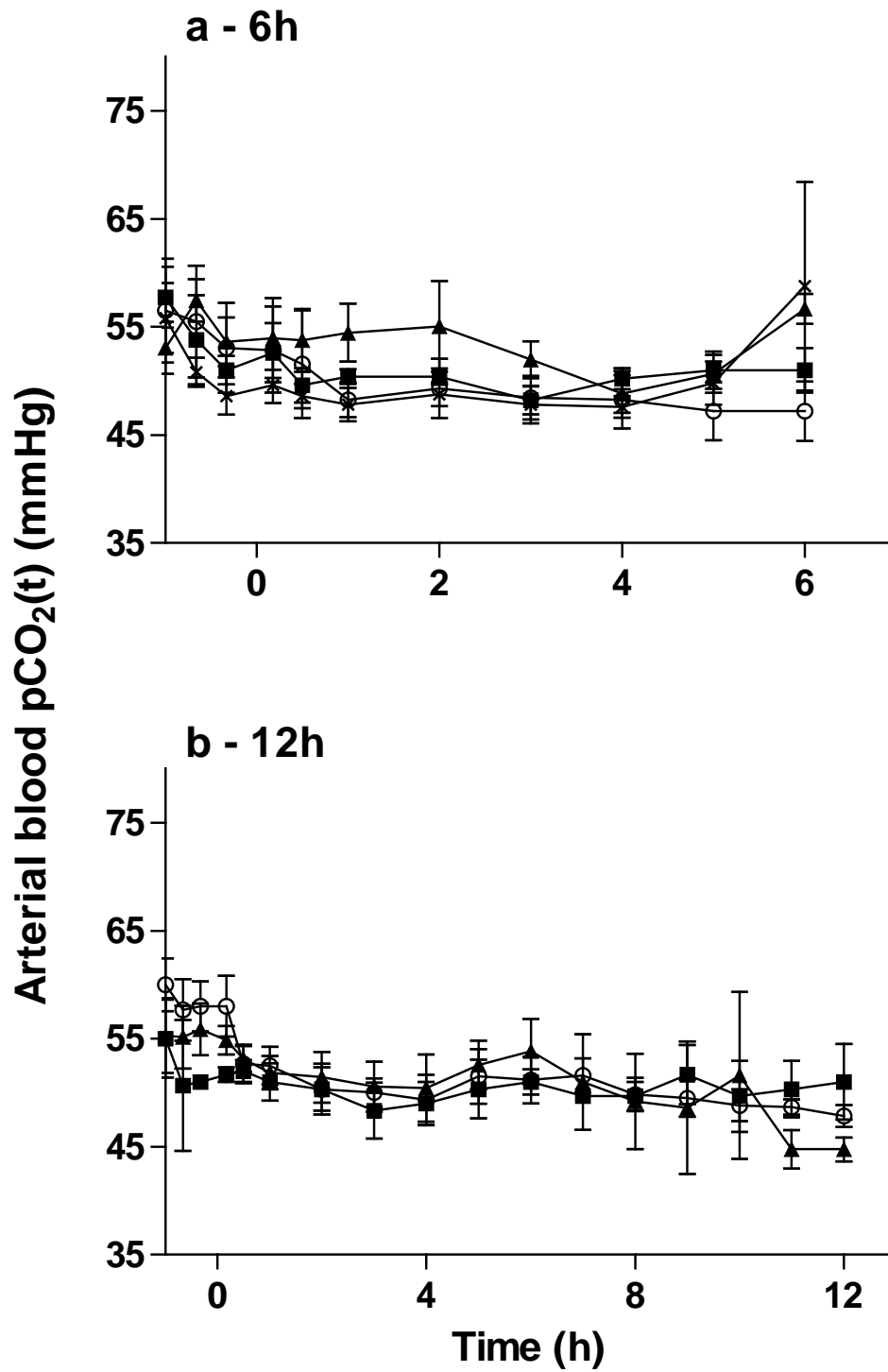
Changes in arterial blood MetHb following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



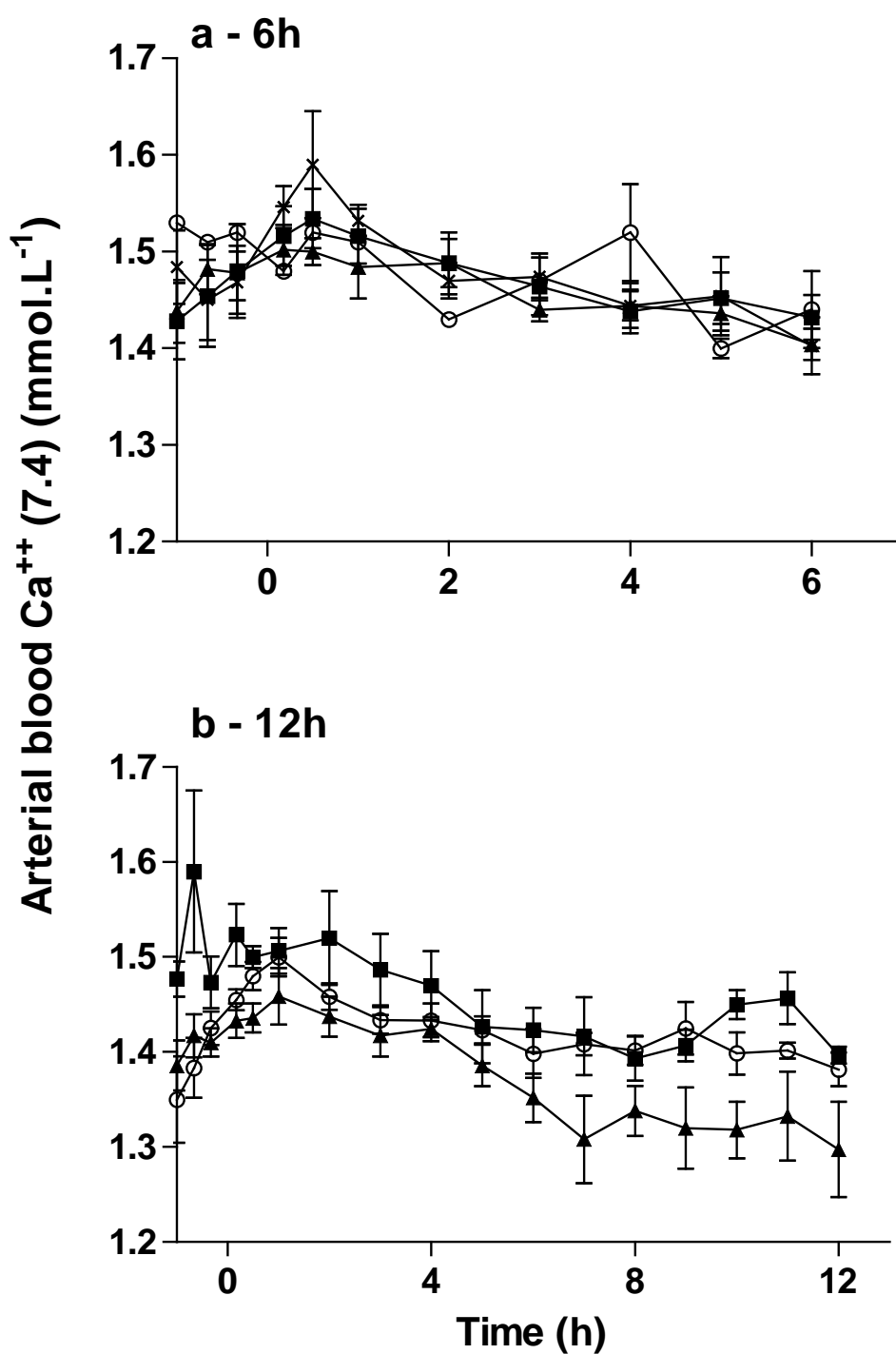
Changes in arterial blood HHb following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



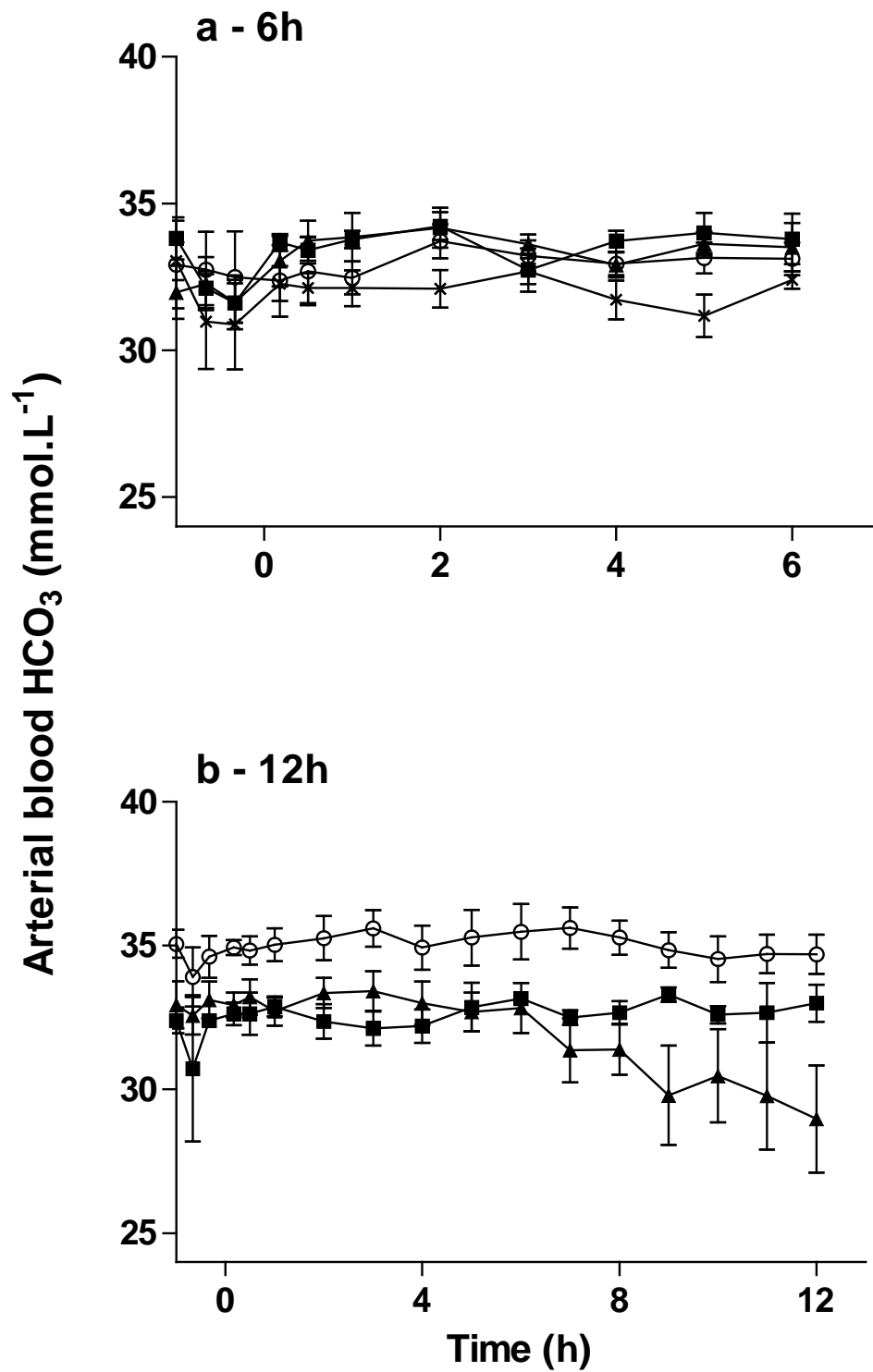
Changes in arterial blood pH (temperature corrected) following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



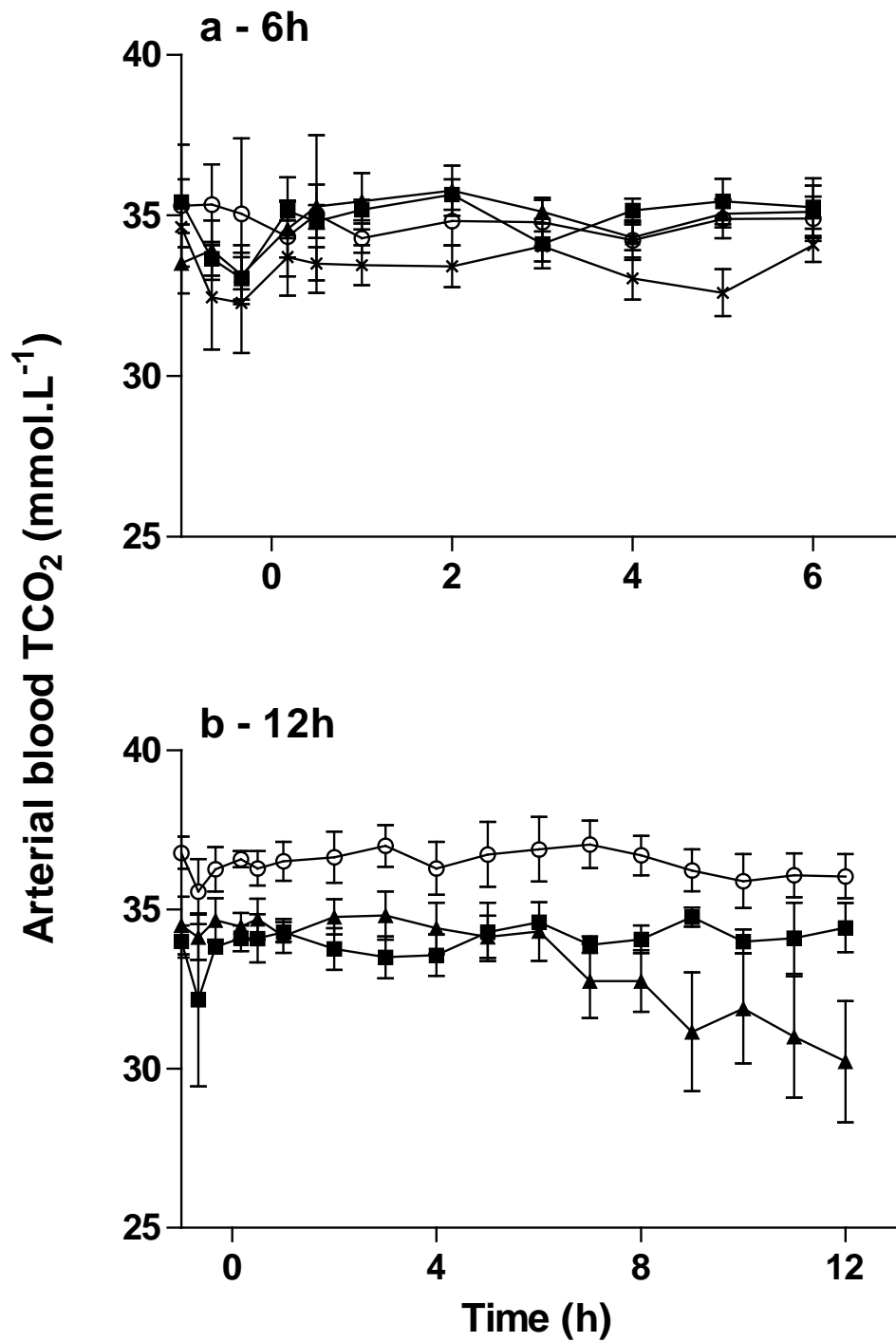
Changes in arterial blood pCO₂ (temperature corrected) following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean ± SE.



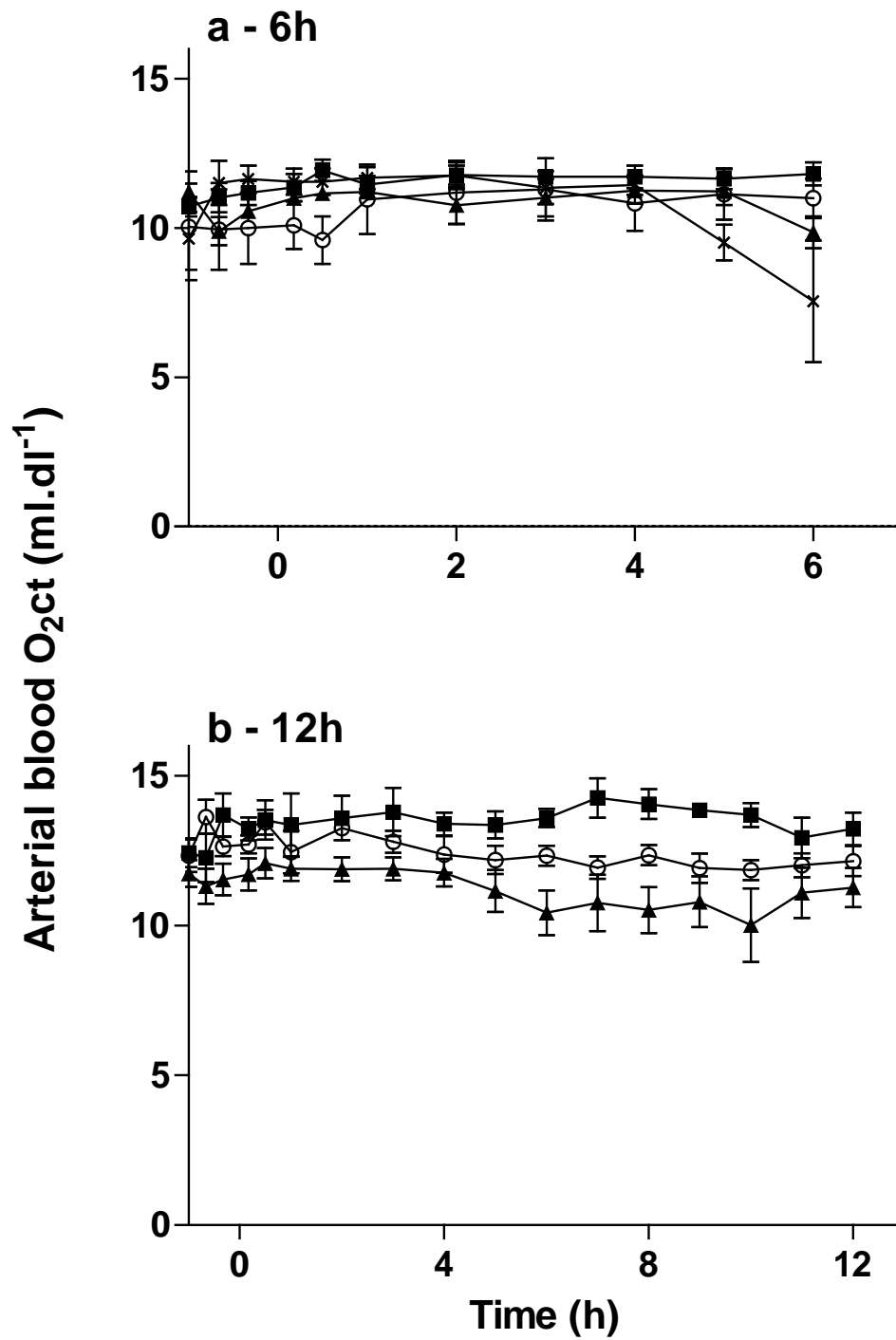
Changes in arterial blood calcium (corrected to pH 7.4) following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



Changes in arterial blood bicarbonate following exposure to low (■), medium (▲) or high (×) doses of HD, or exposure to air (○). Data are expressed as mean \pm SE.

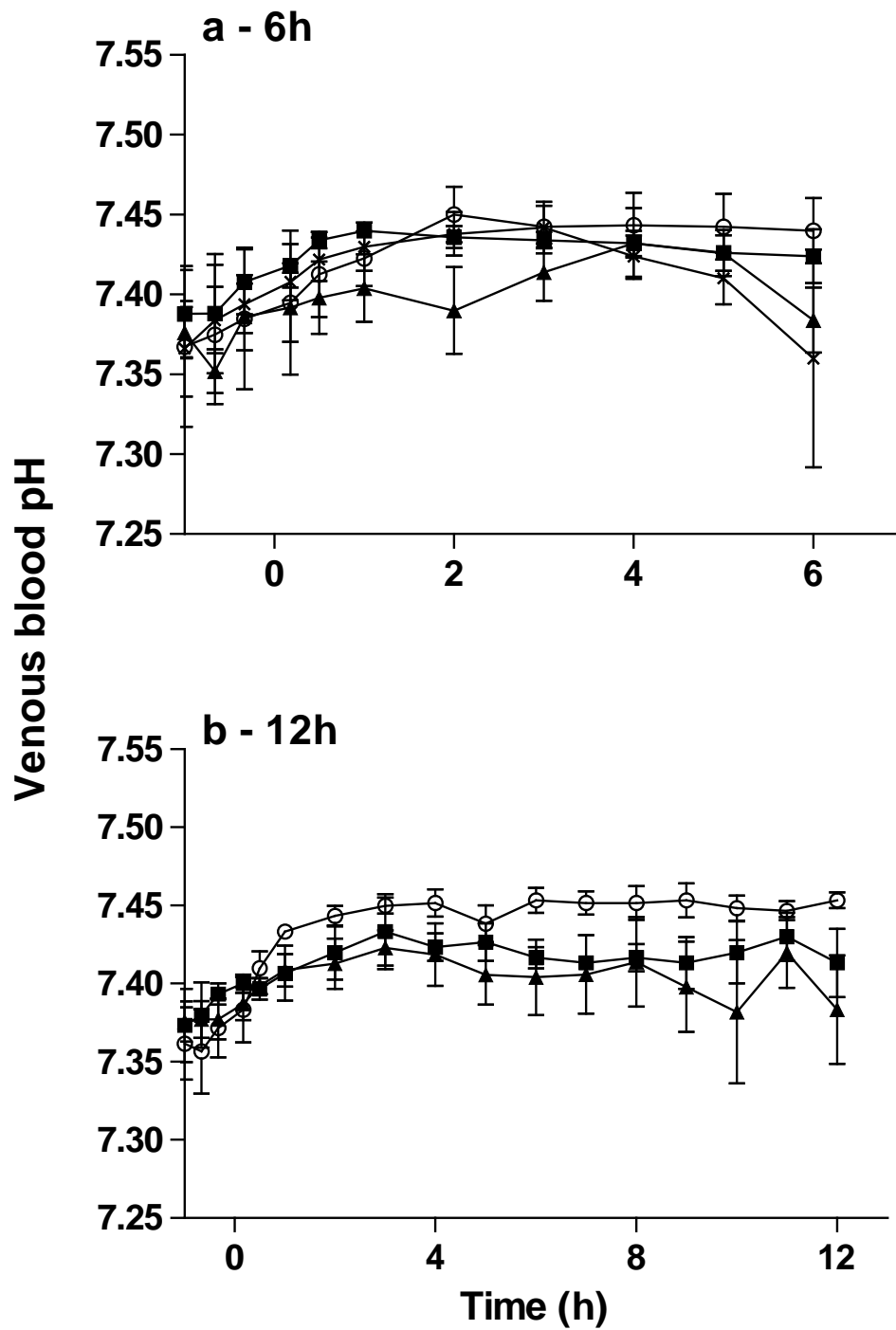


Changes in arterial blood TCO₂ following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.

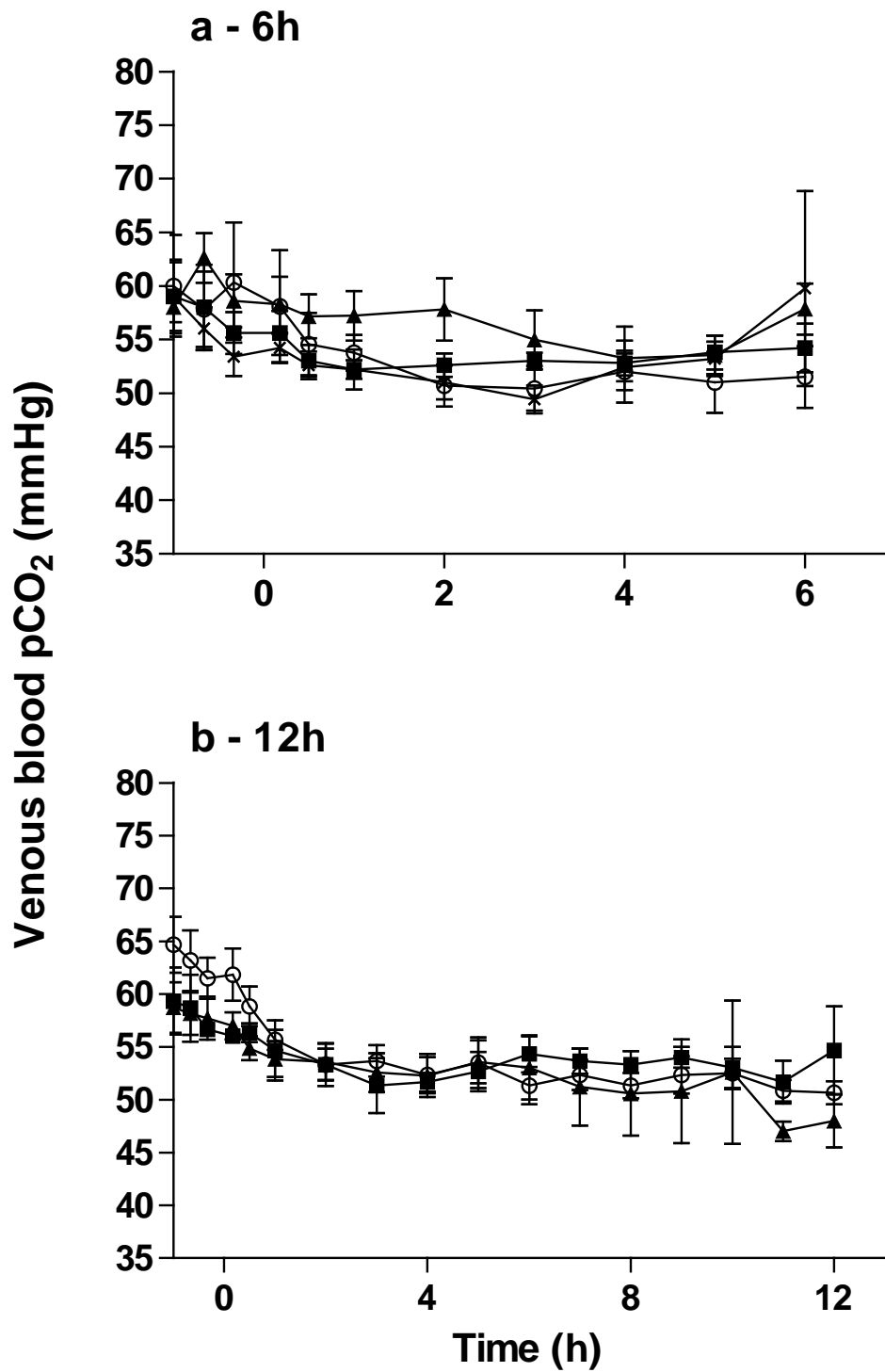


Changes in arterial blood O₂ct following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean ± SE.

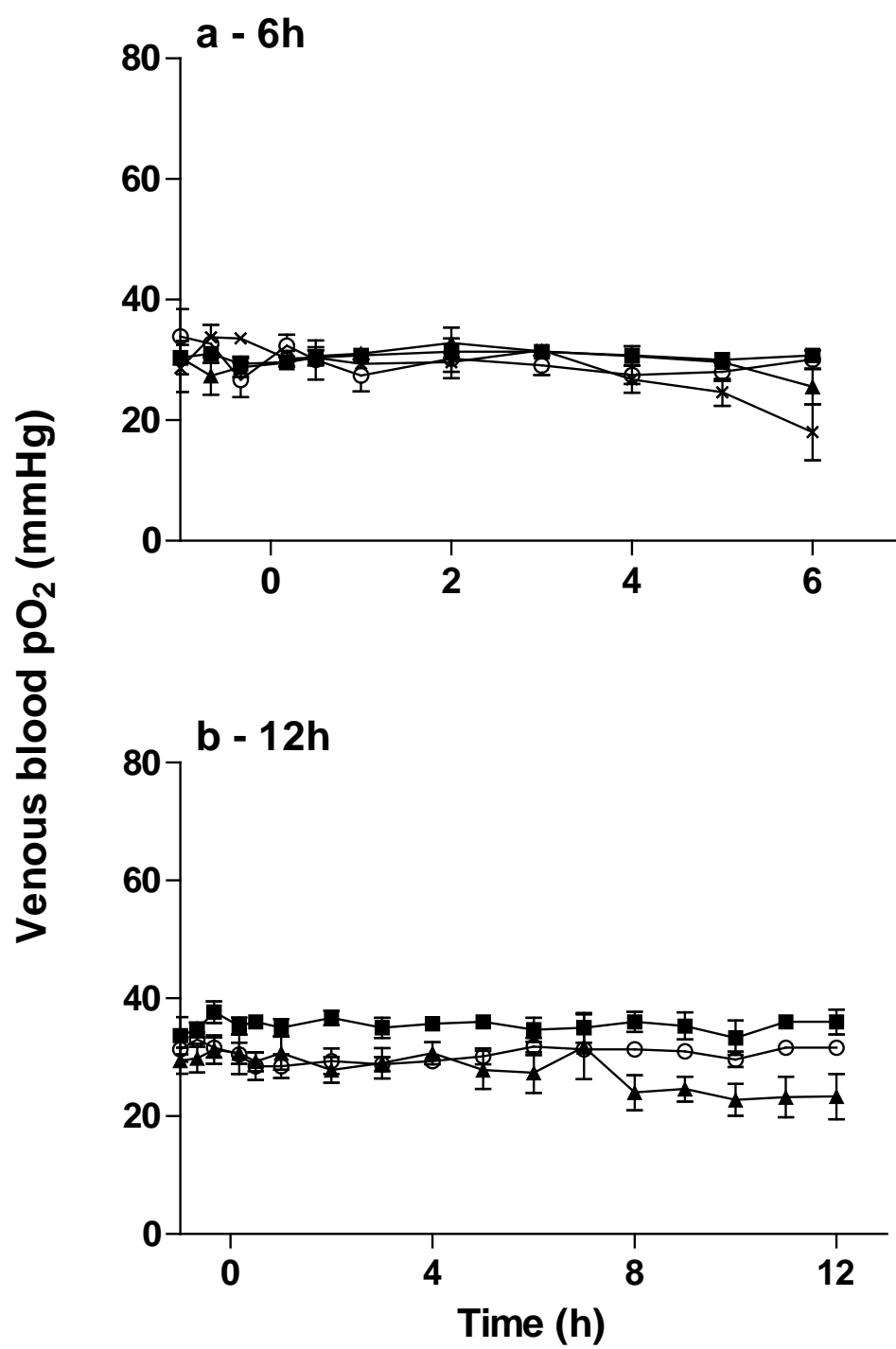
Blood Gas Parameters – Venous

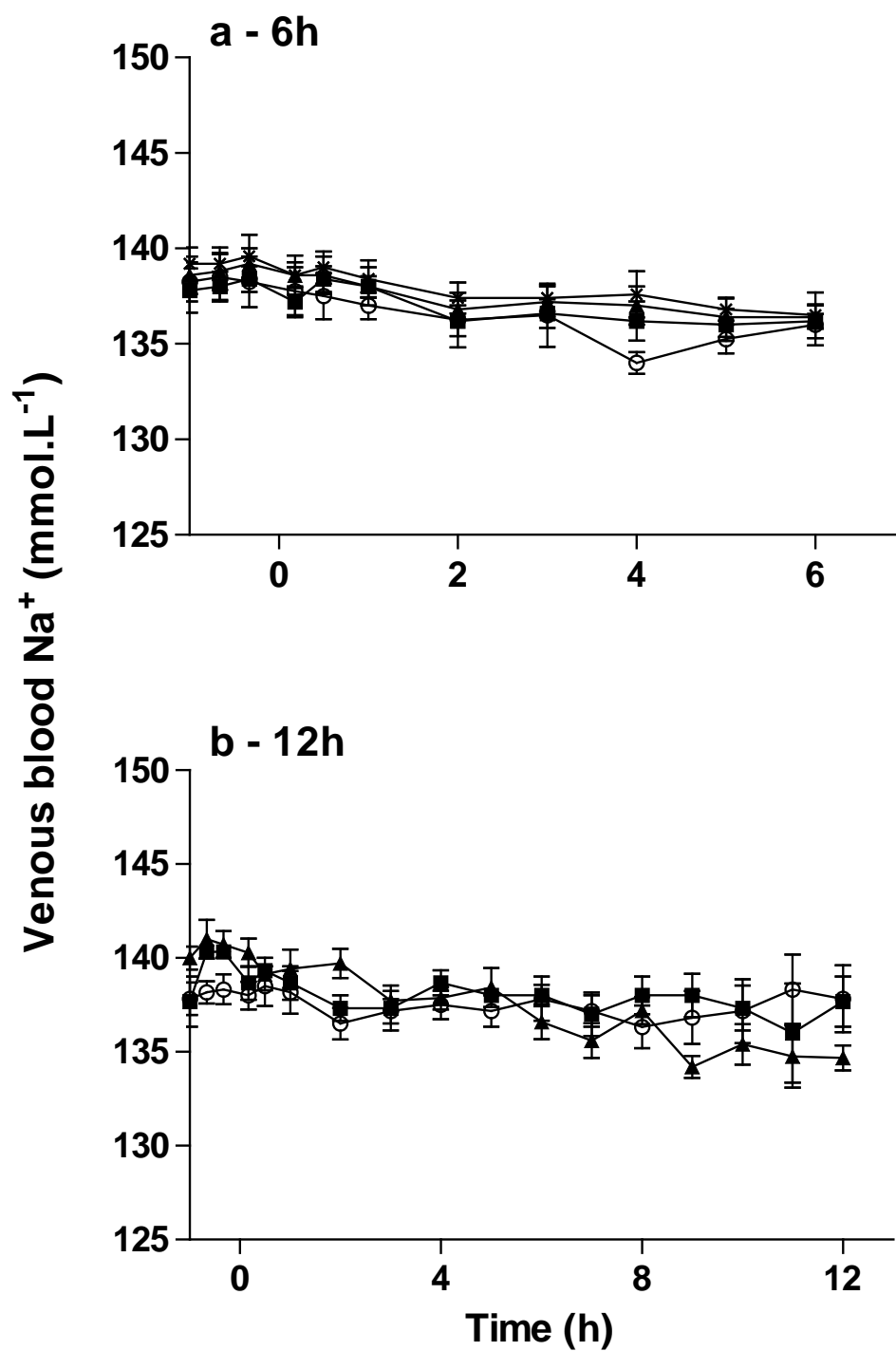


Changes in venous blood pH following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (○). Data are expressed as mean \pm SE.

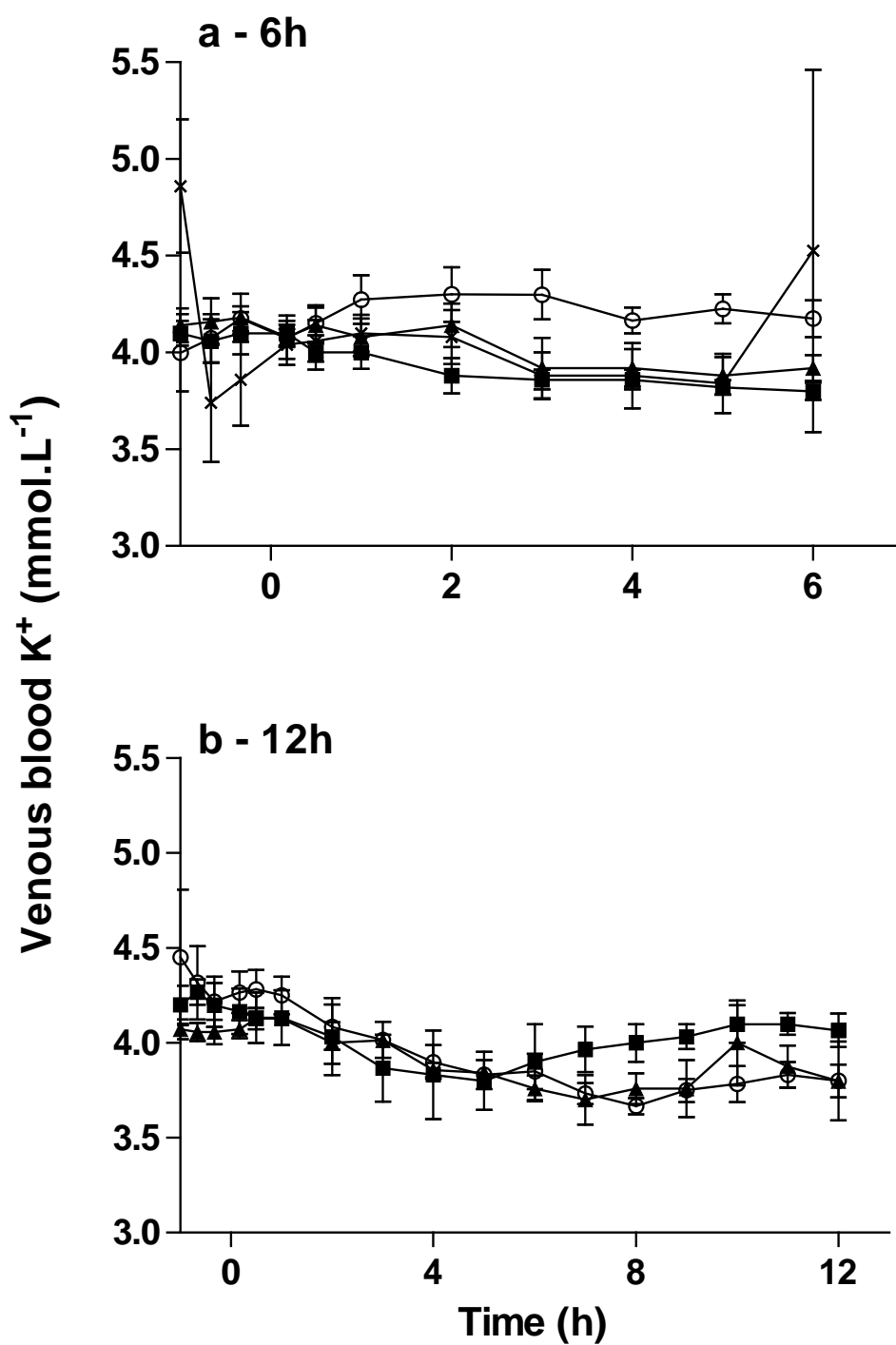


Changes in venous blood pCO₂ following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean ± SE.

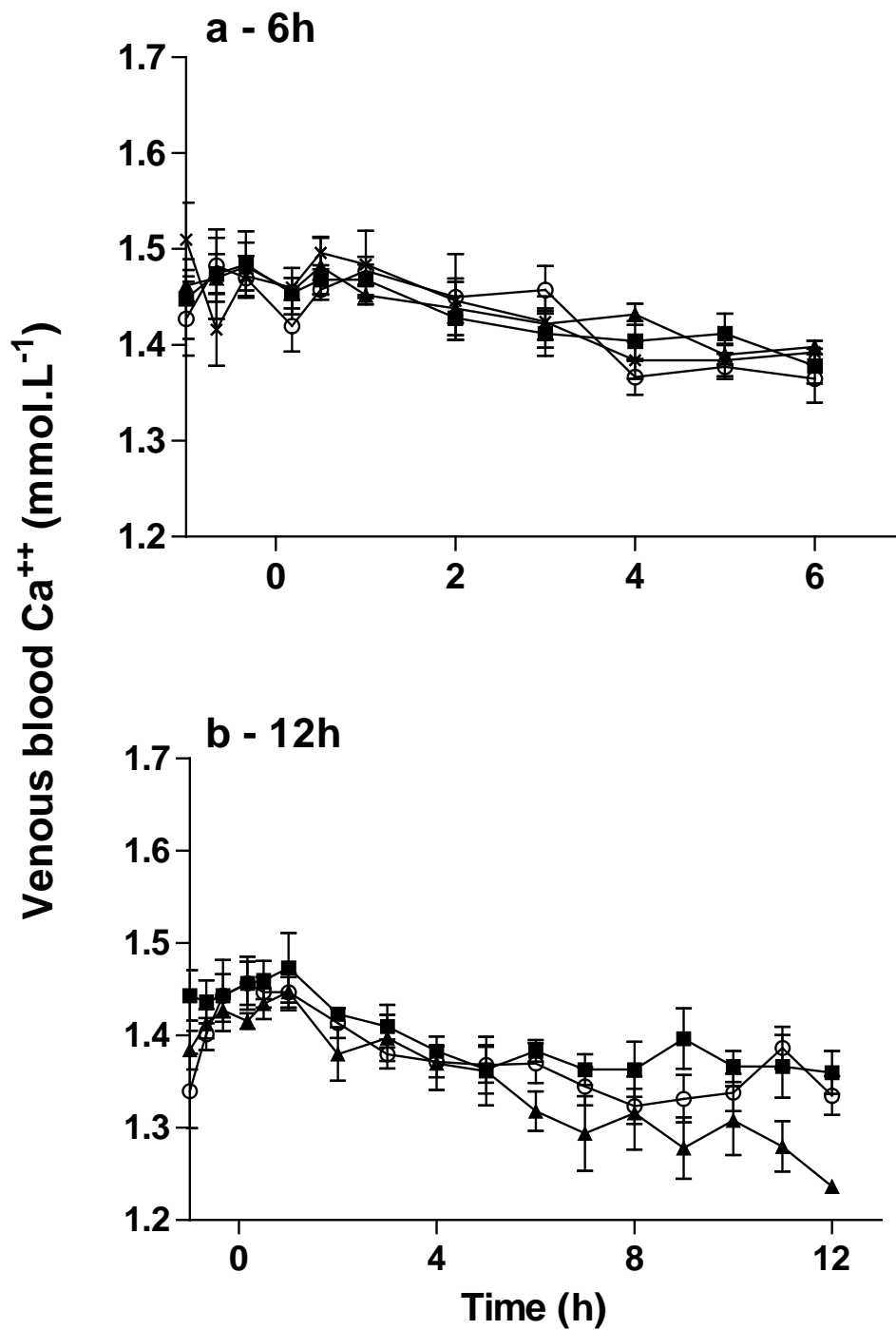




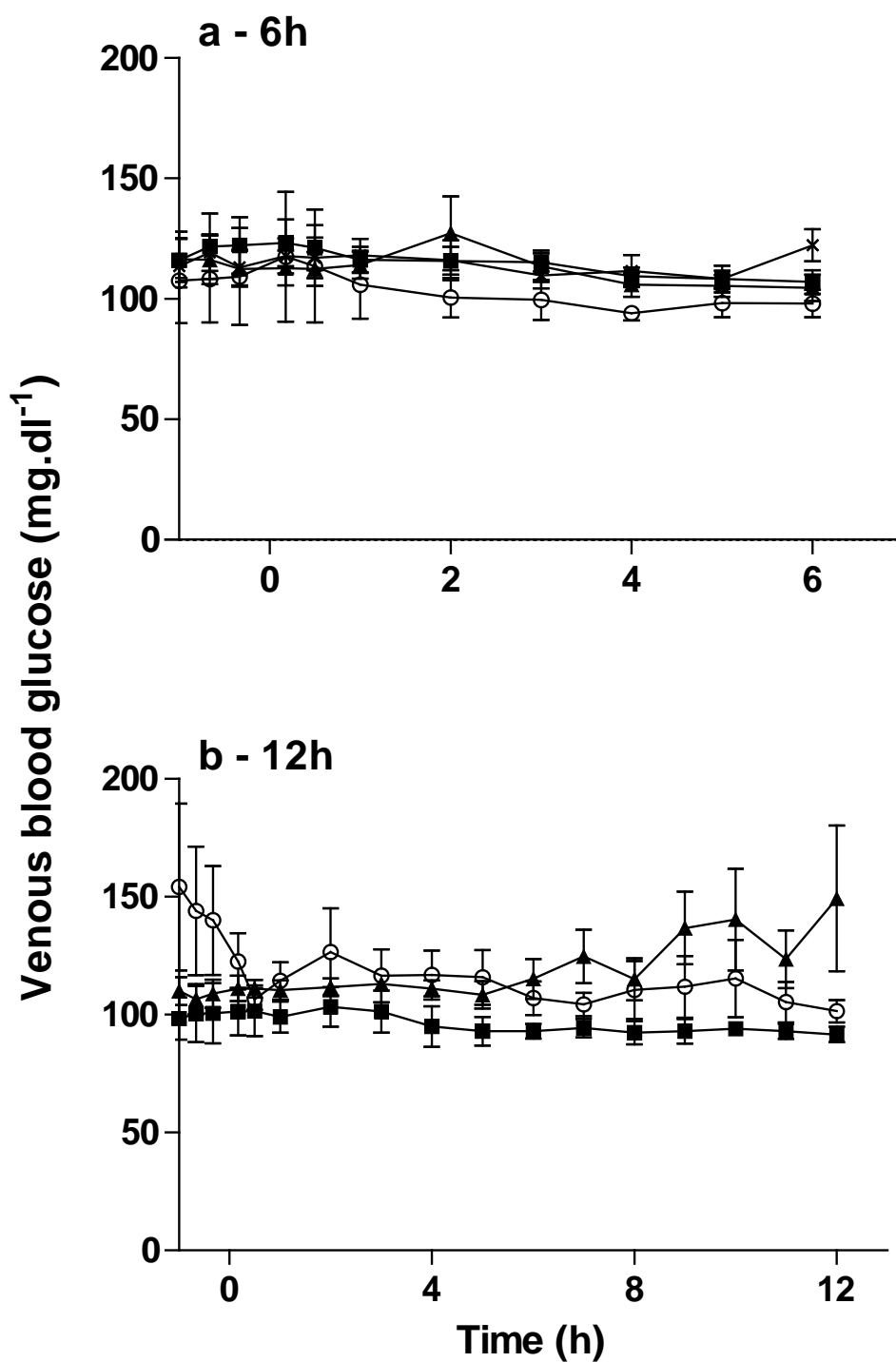
Changes in venous blood sodium following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



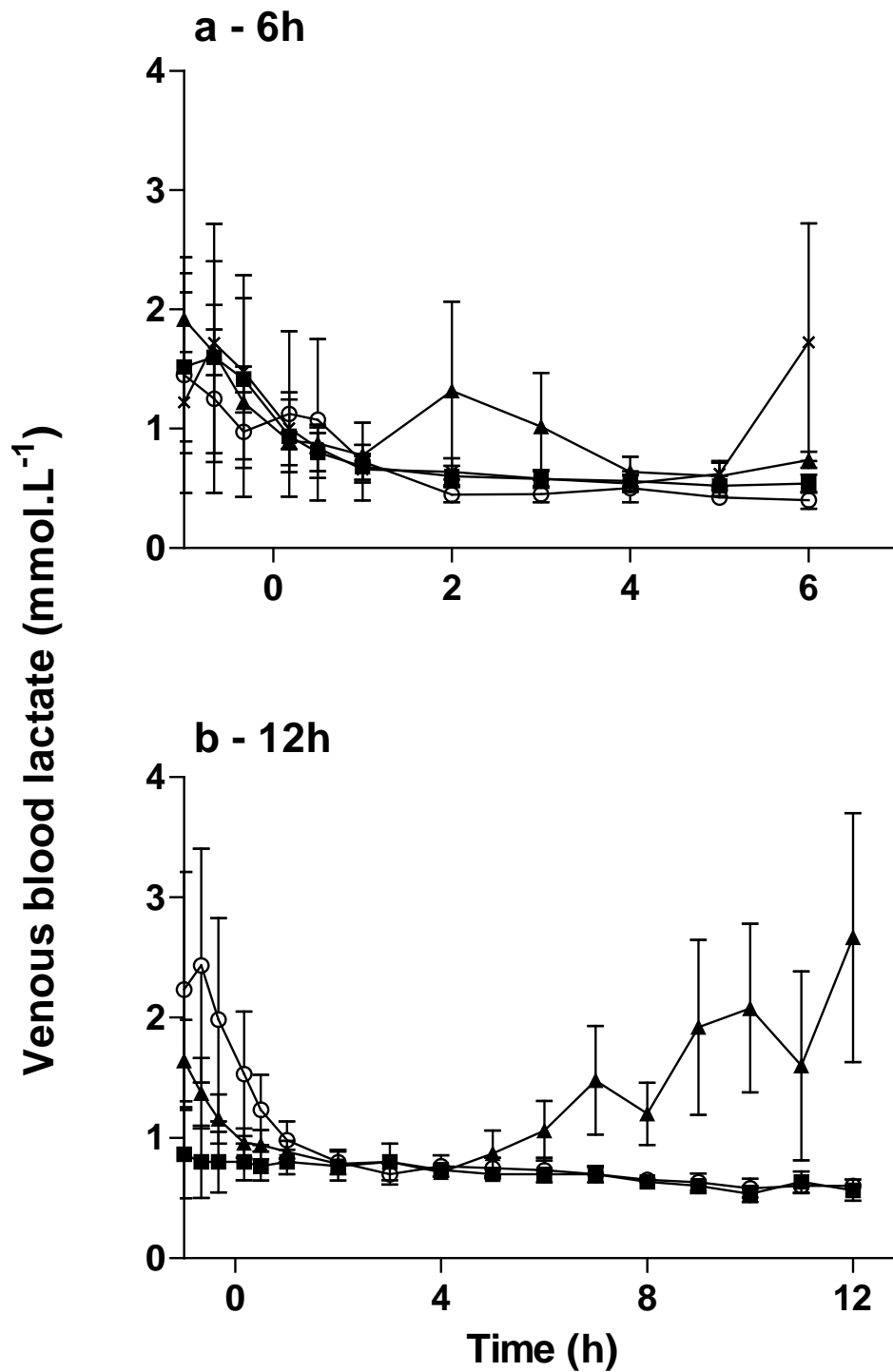
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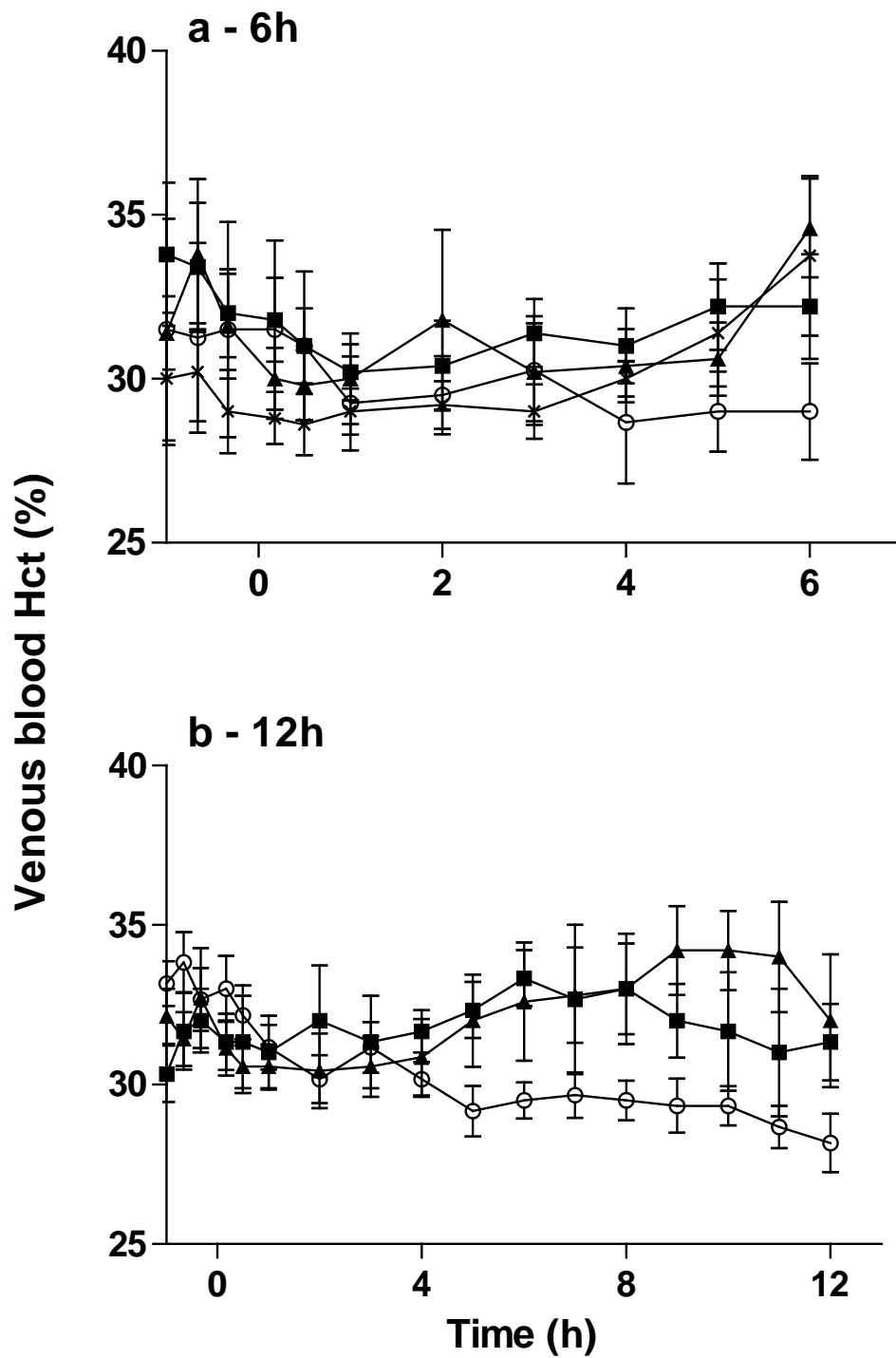
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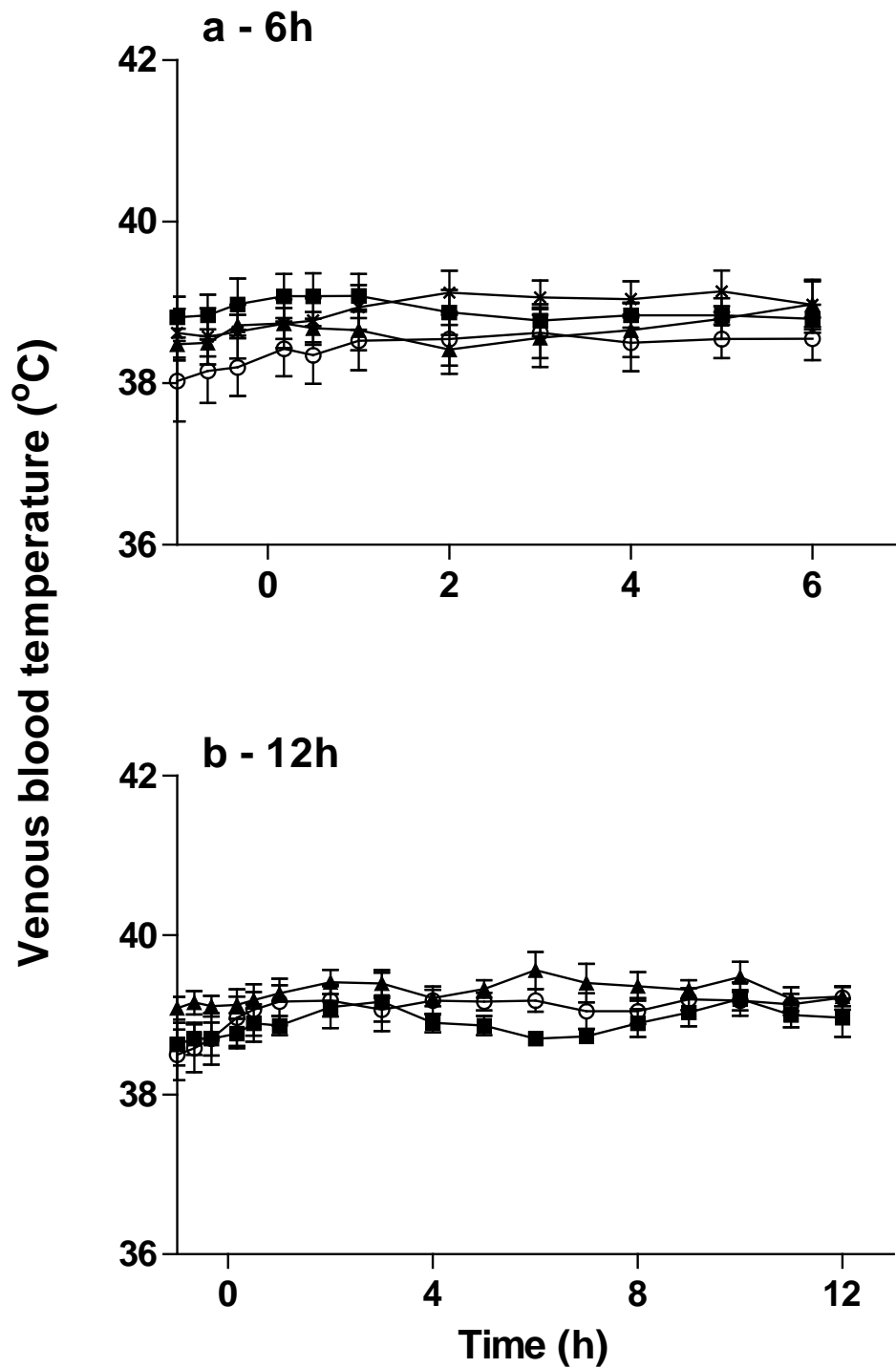
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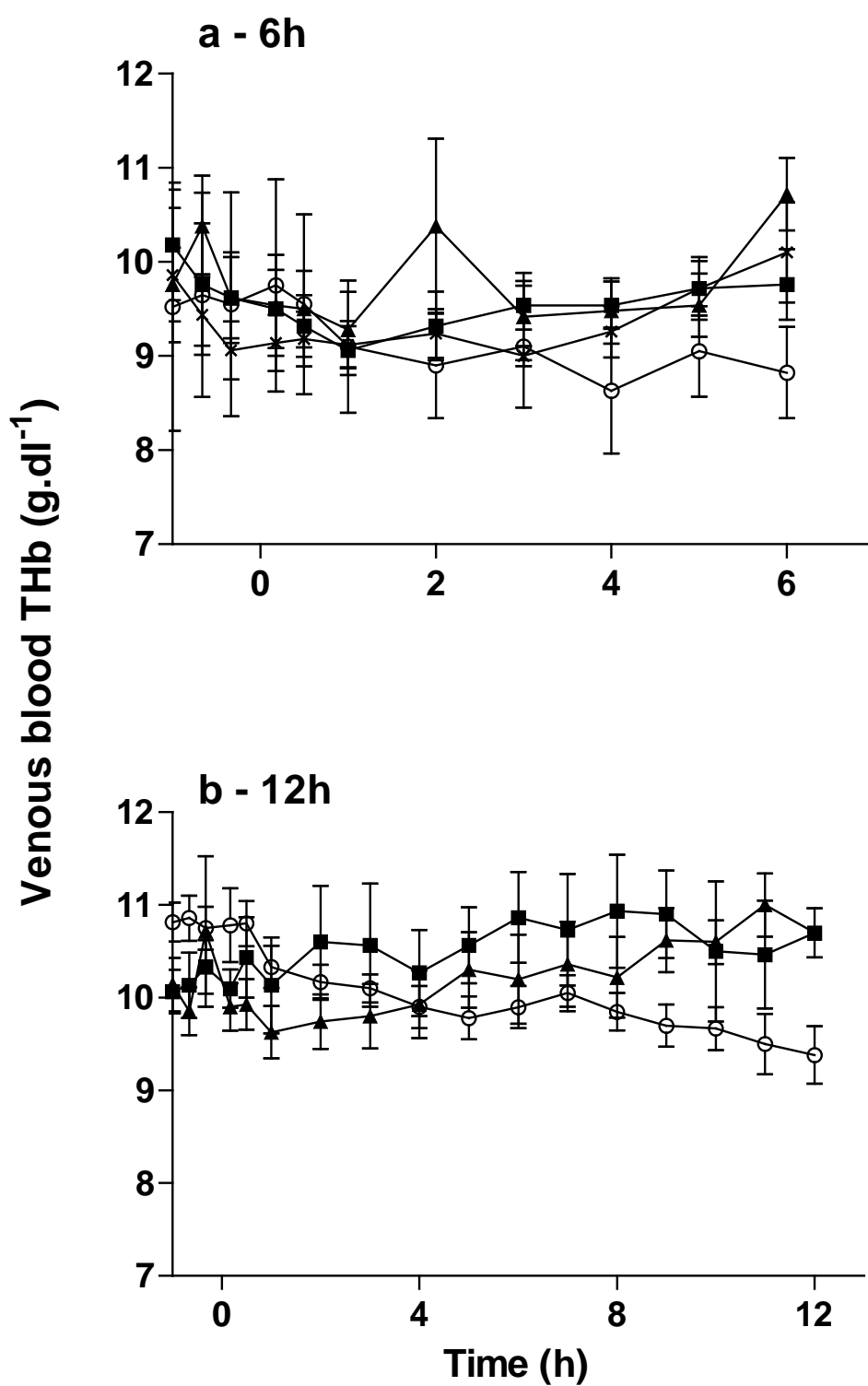
Changes in venous blood lactate following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



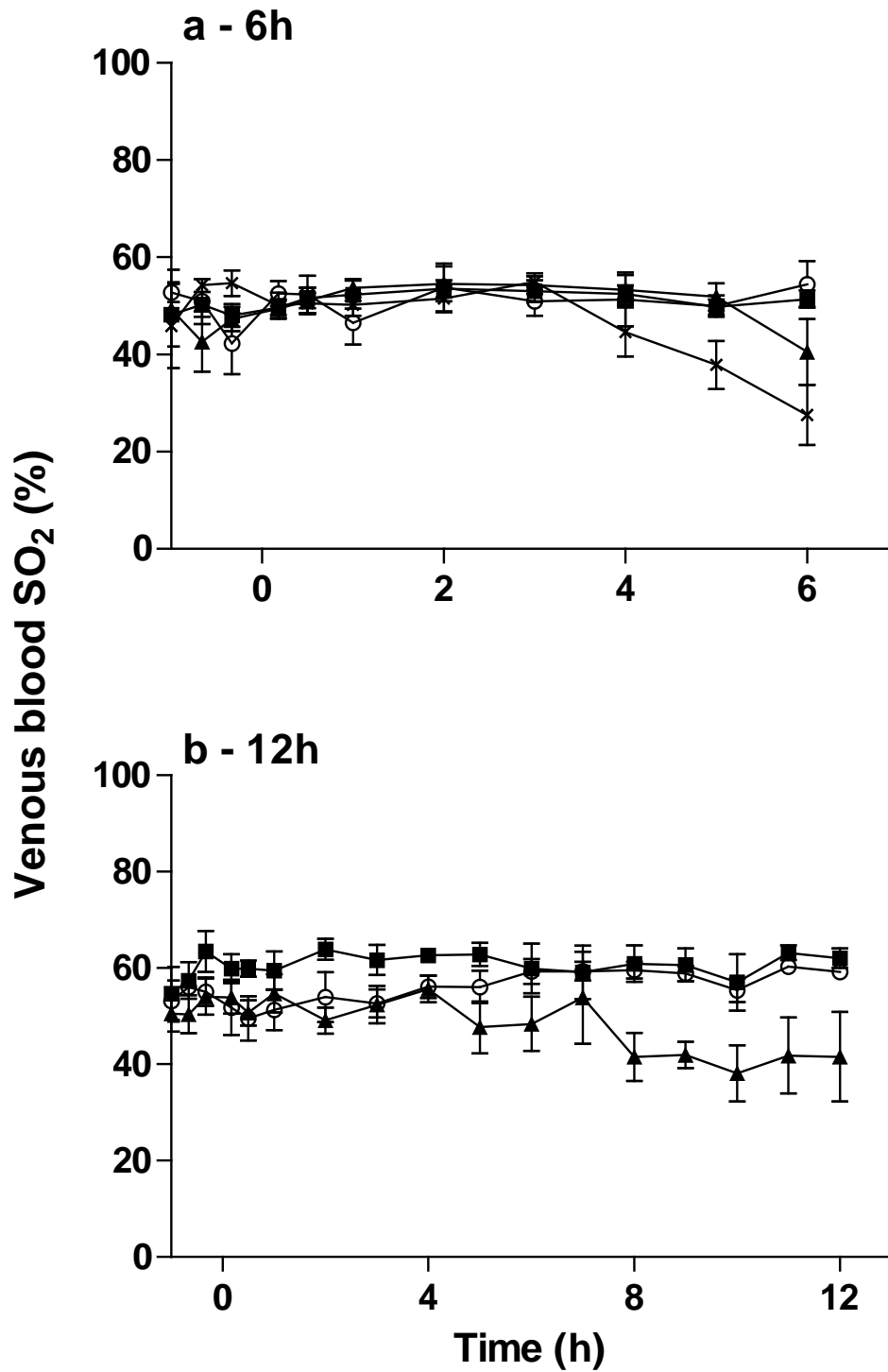
Changes in venous blood hematocrit following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (○). Data are expressed as mean \pm SE.



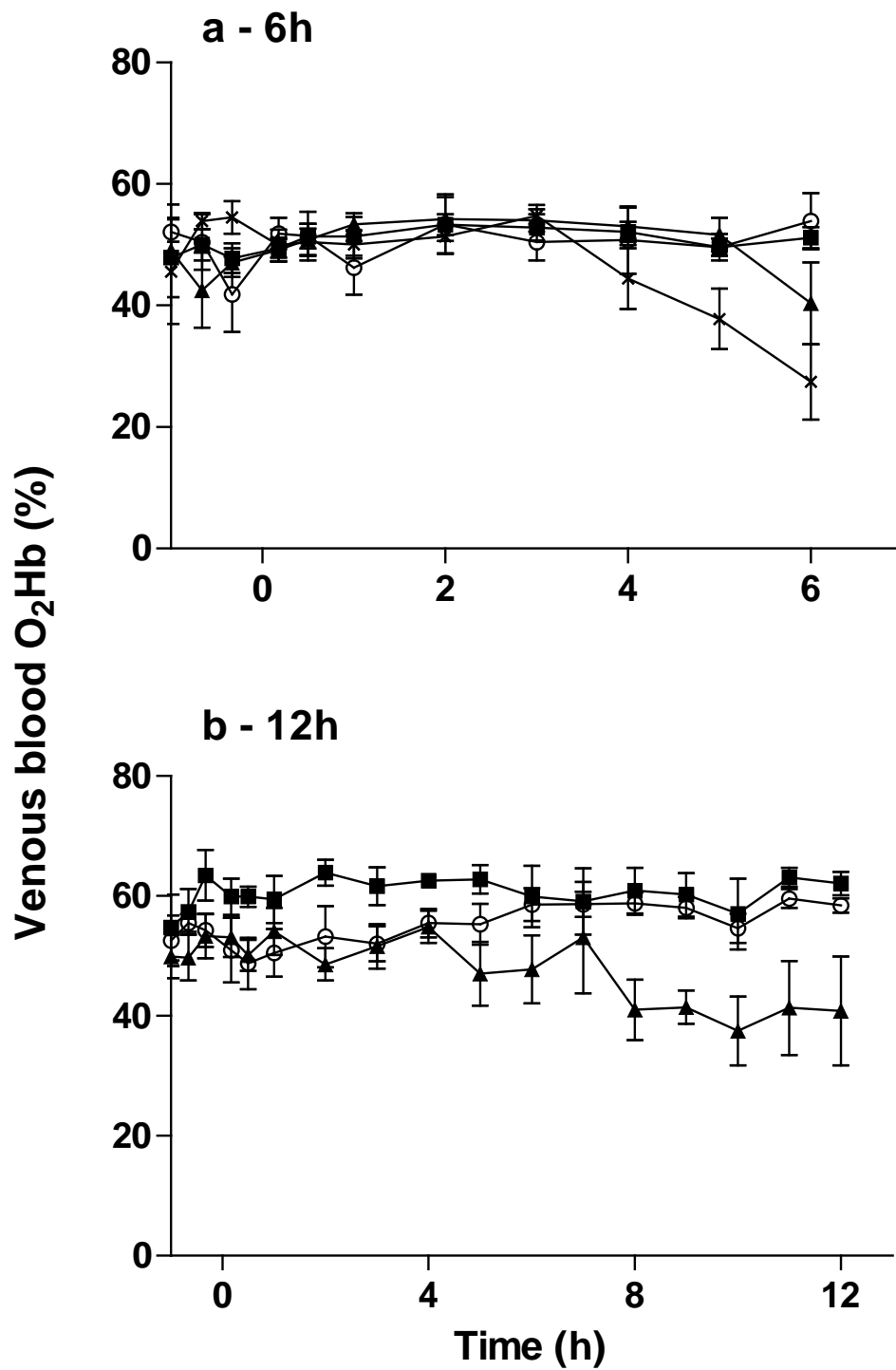
Changes in venous blood temperature following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean ± SE.



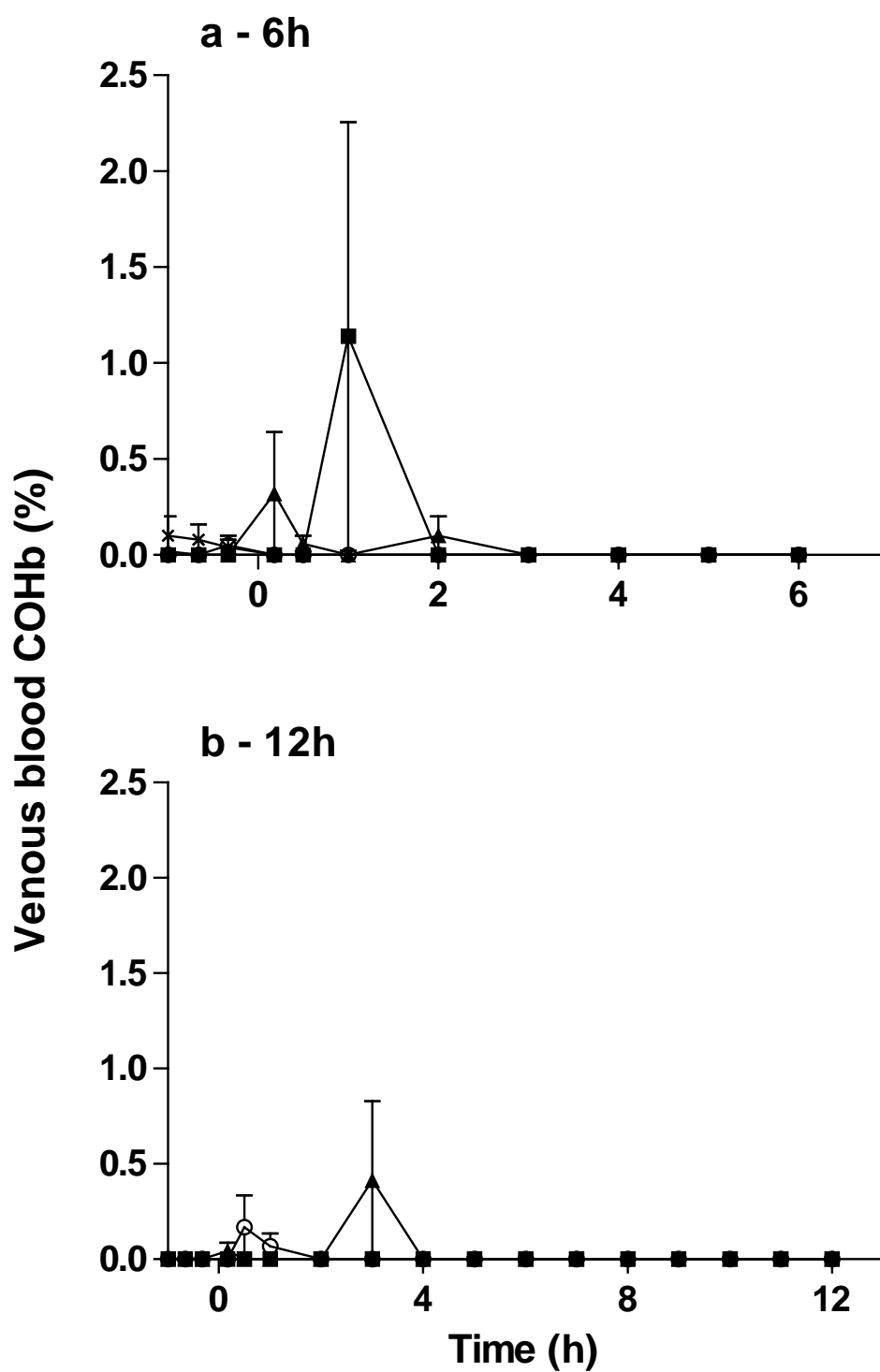
Changes in venous blood THb following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



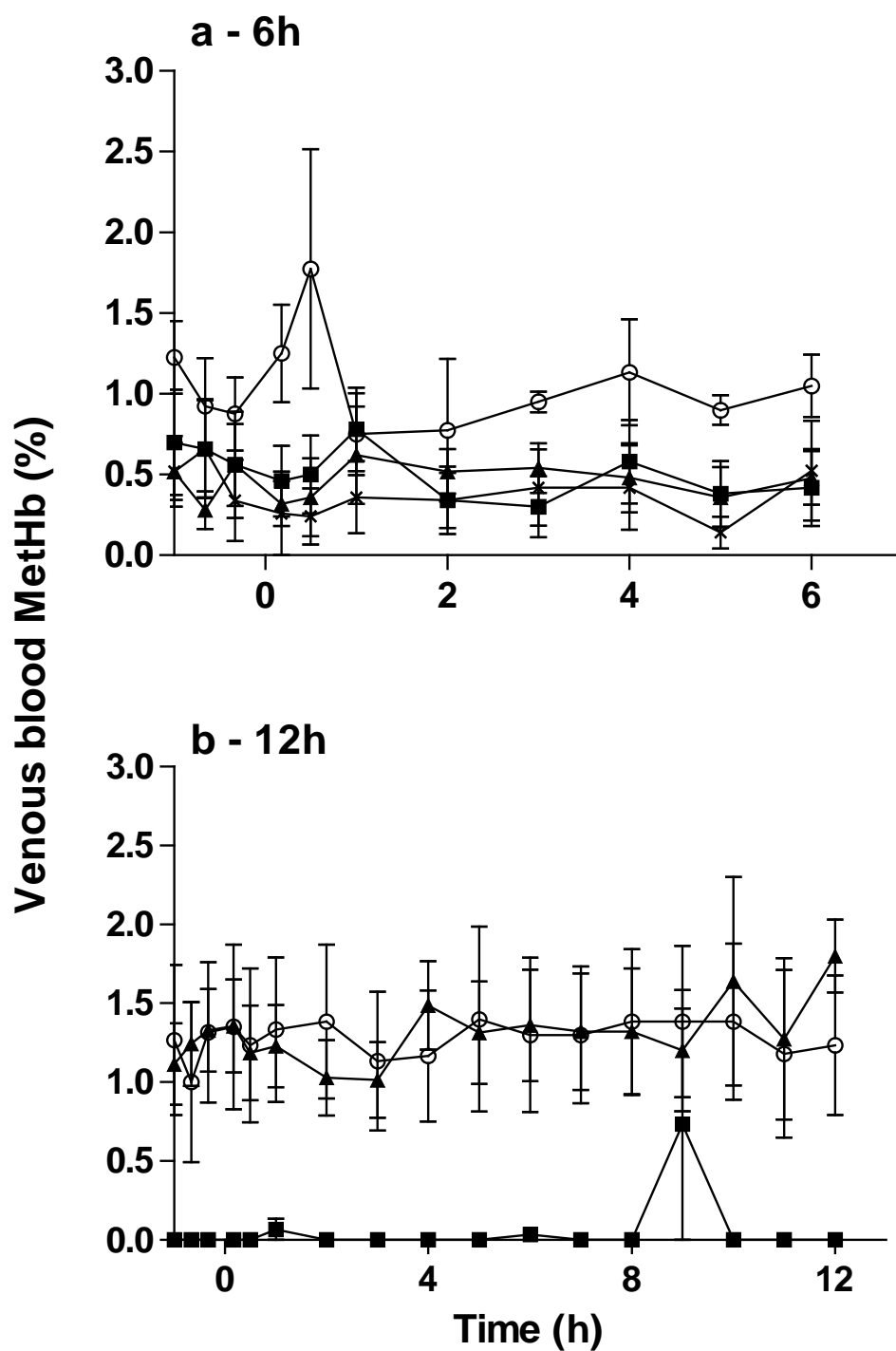
Changes in venous blood SO_2 following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



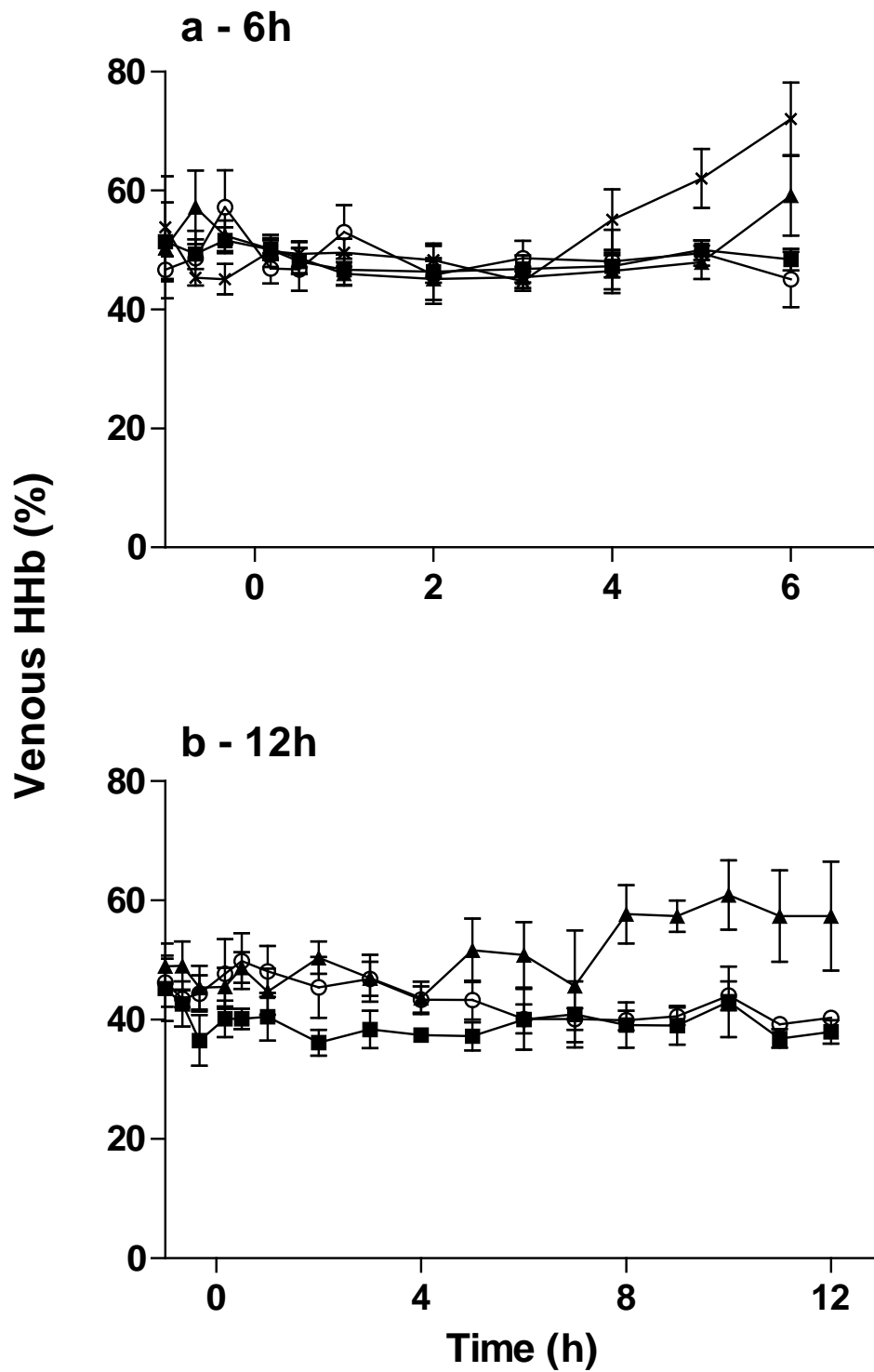
Changes in venous blood O₂Hb following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



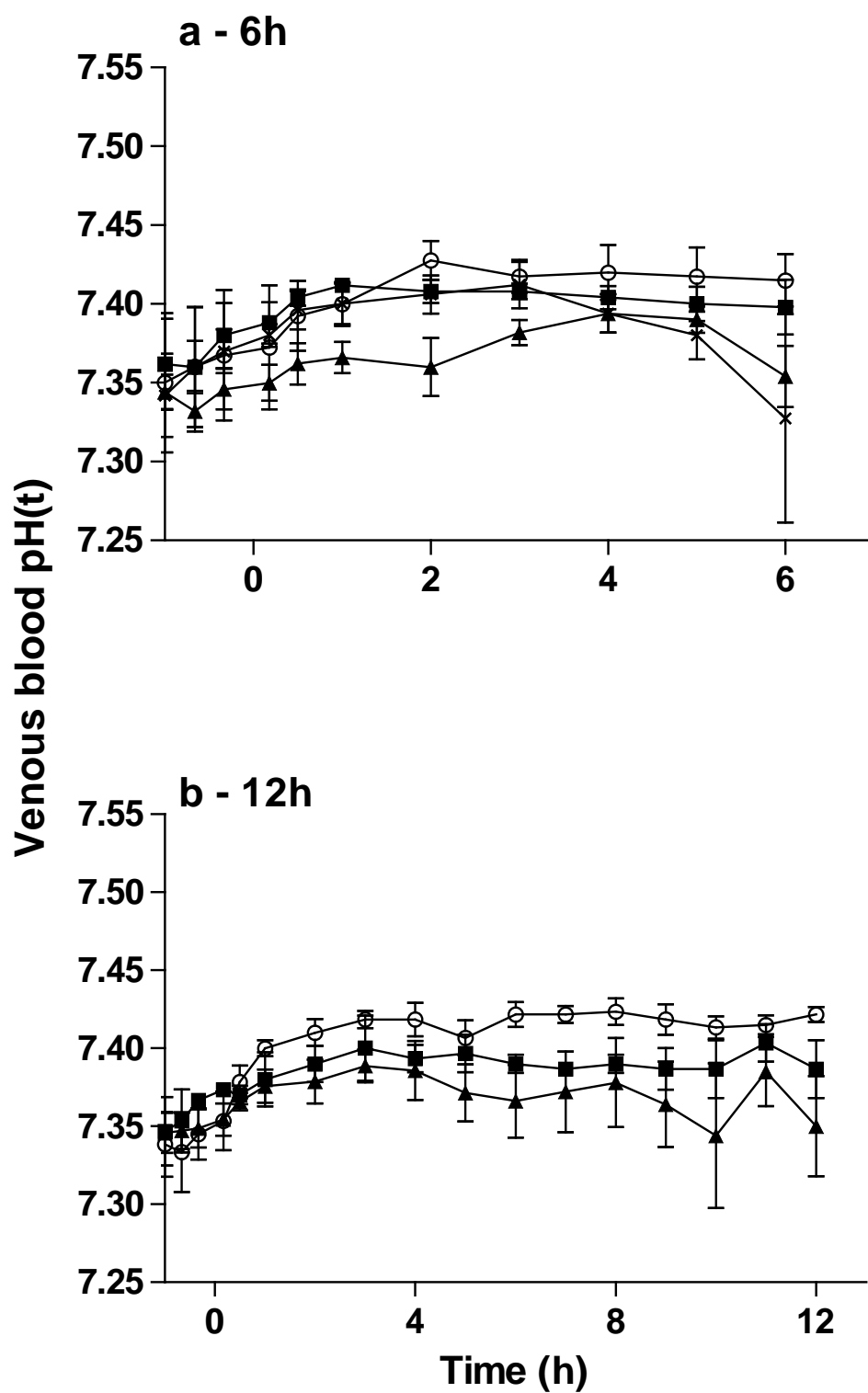
Changes in venous blood COHb following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



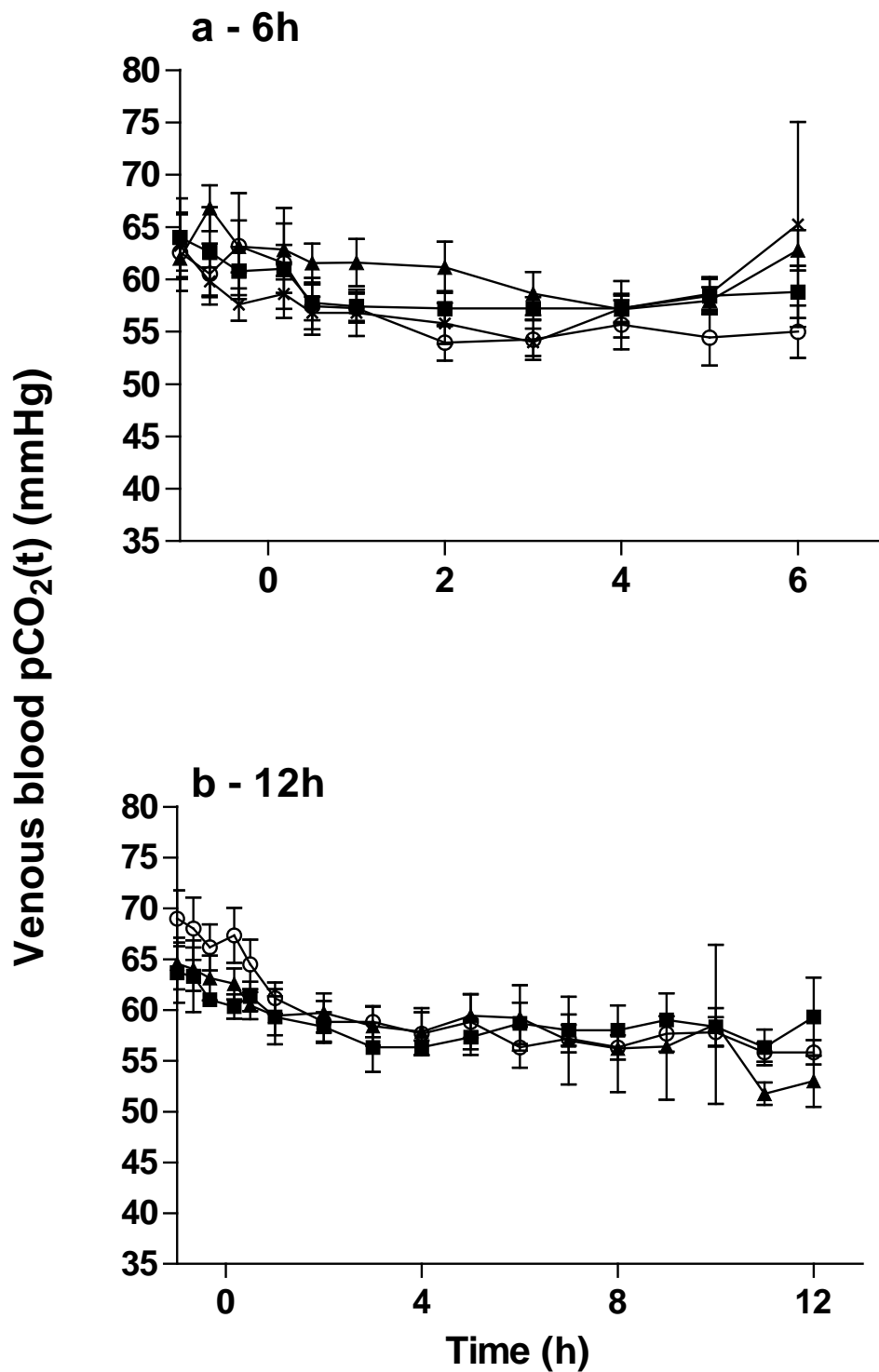
Changes in venous blood MetHb following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (○). Data are expressed as mean \pm SE.



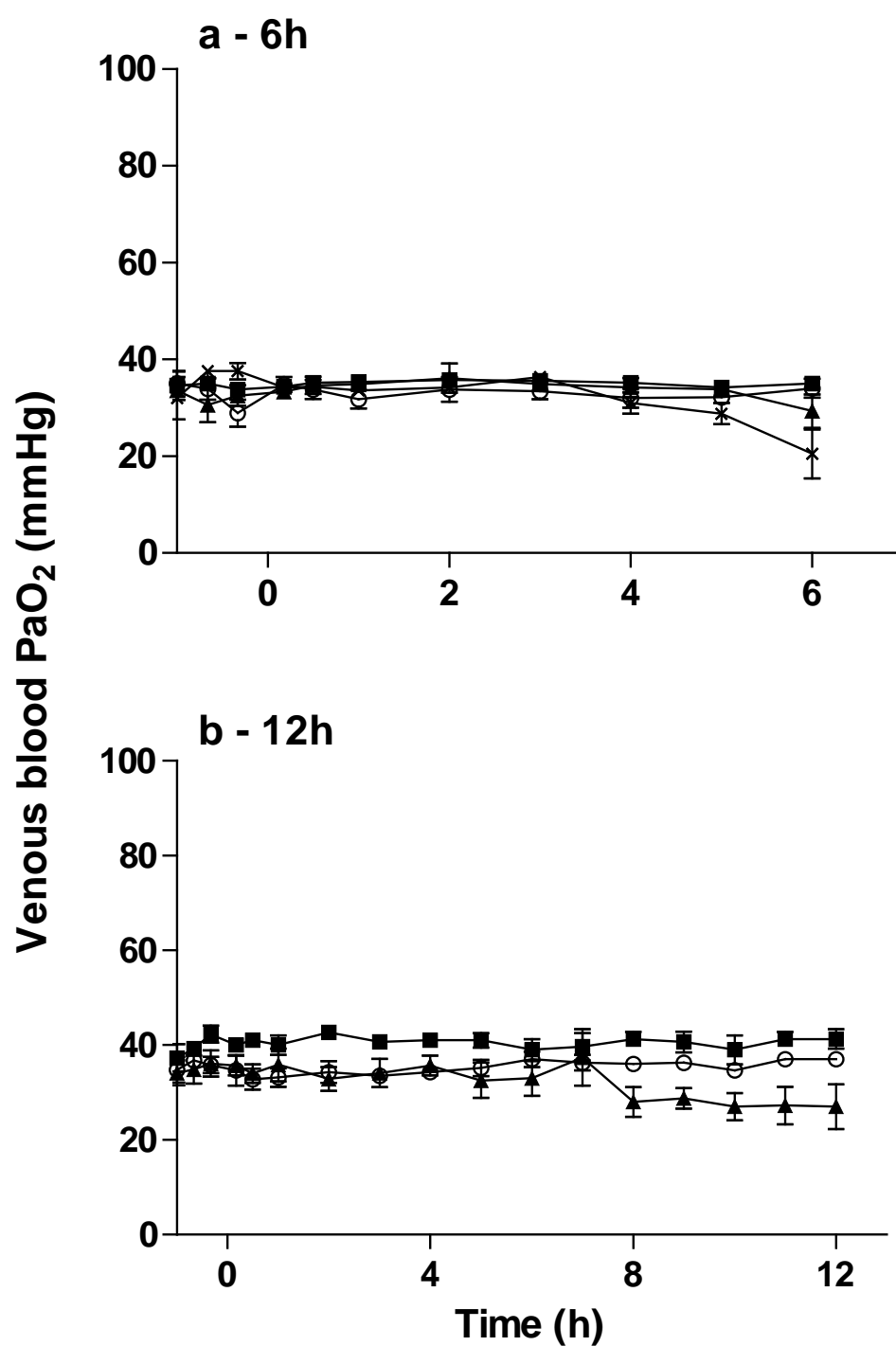
Changes in venous blood HHb following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



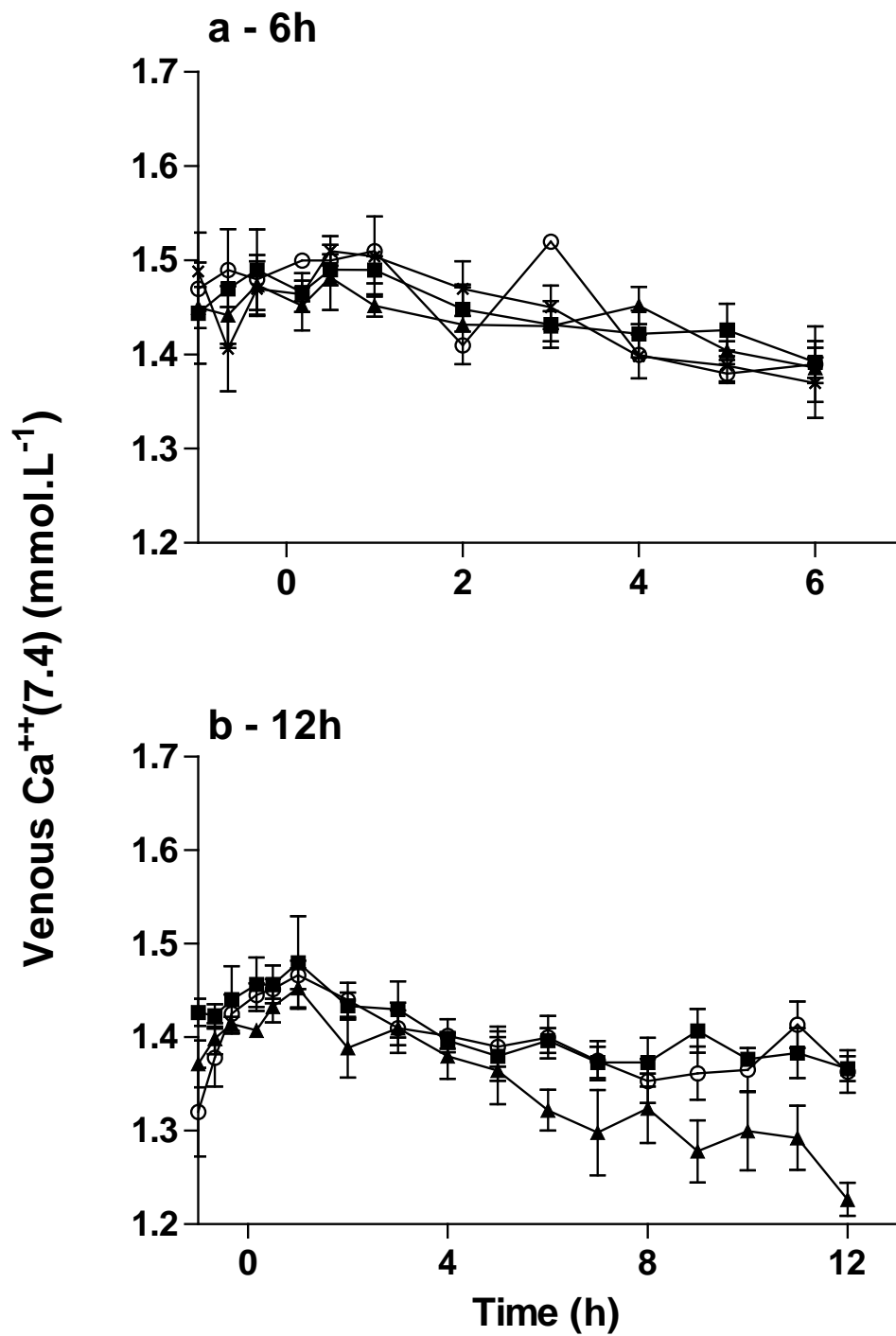
Changes in venous blood pH (temperature corrected) following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (○). Data are expressed as mean \pm SE.



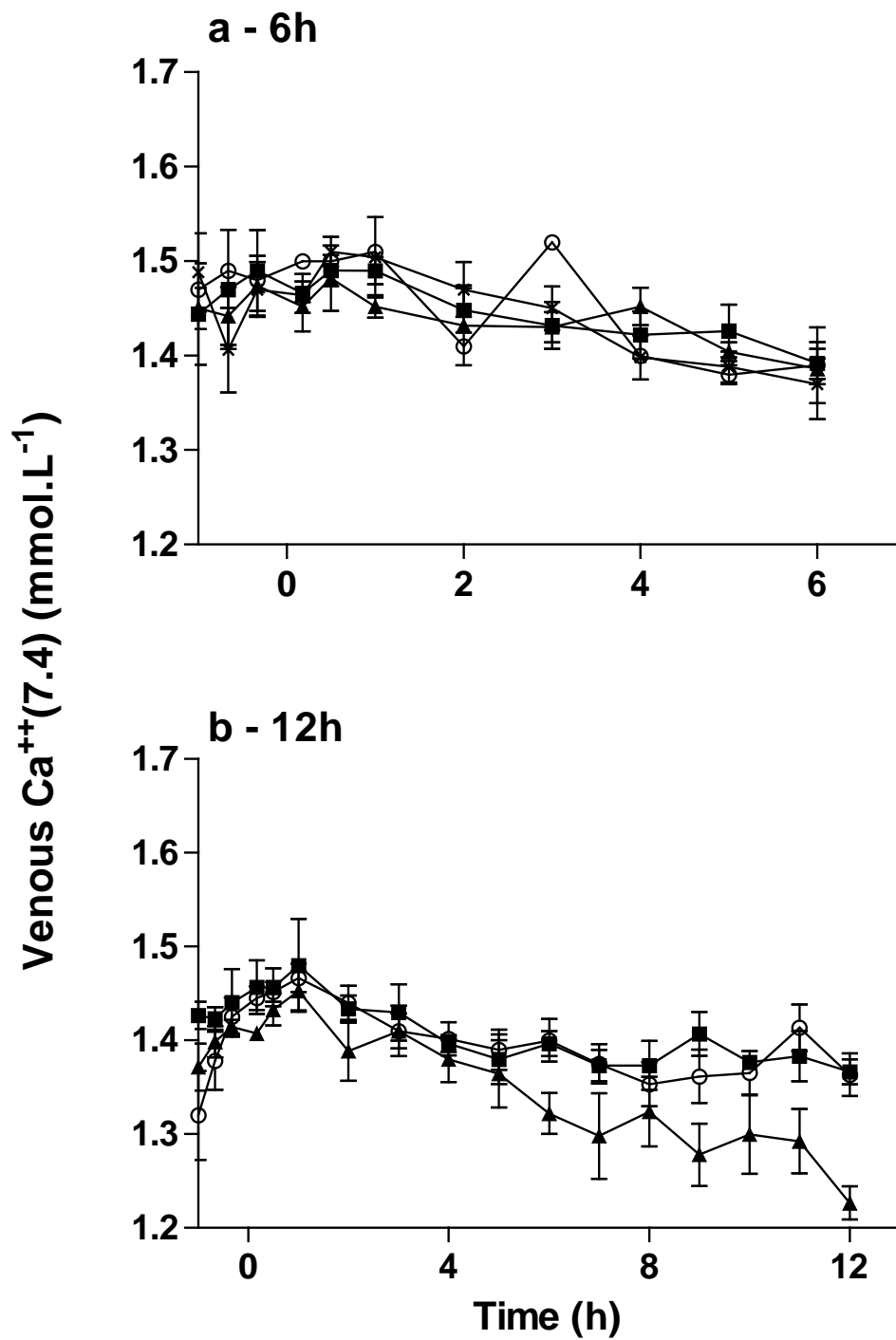
Changes in venous blood $p\text{CO}_2$ (temperature corrected) following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



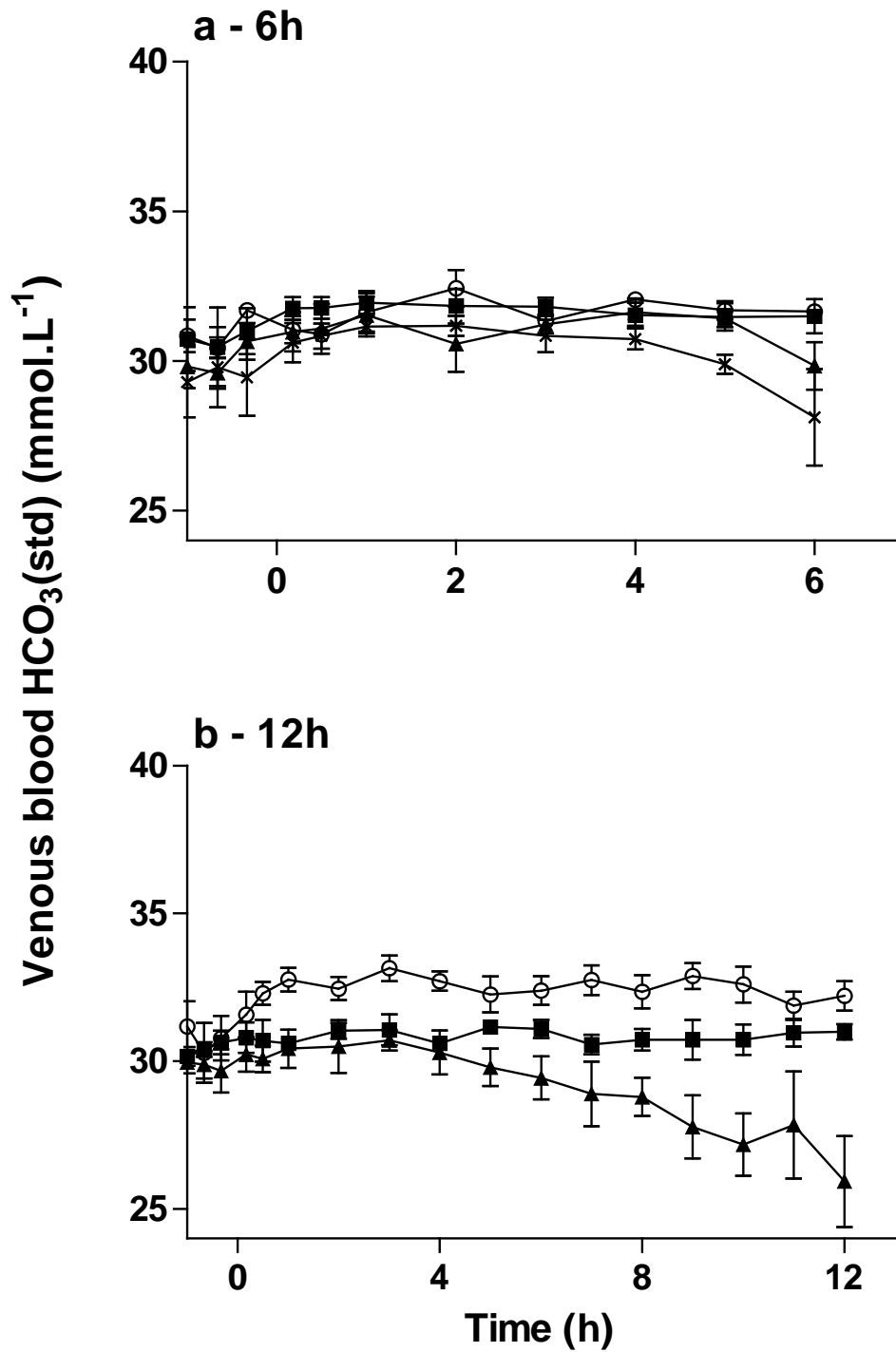
Changes in venous blood PaO₂ following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean ± SE.



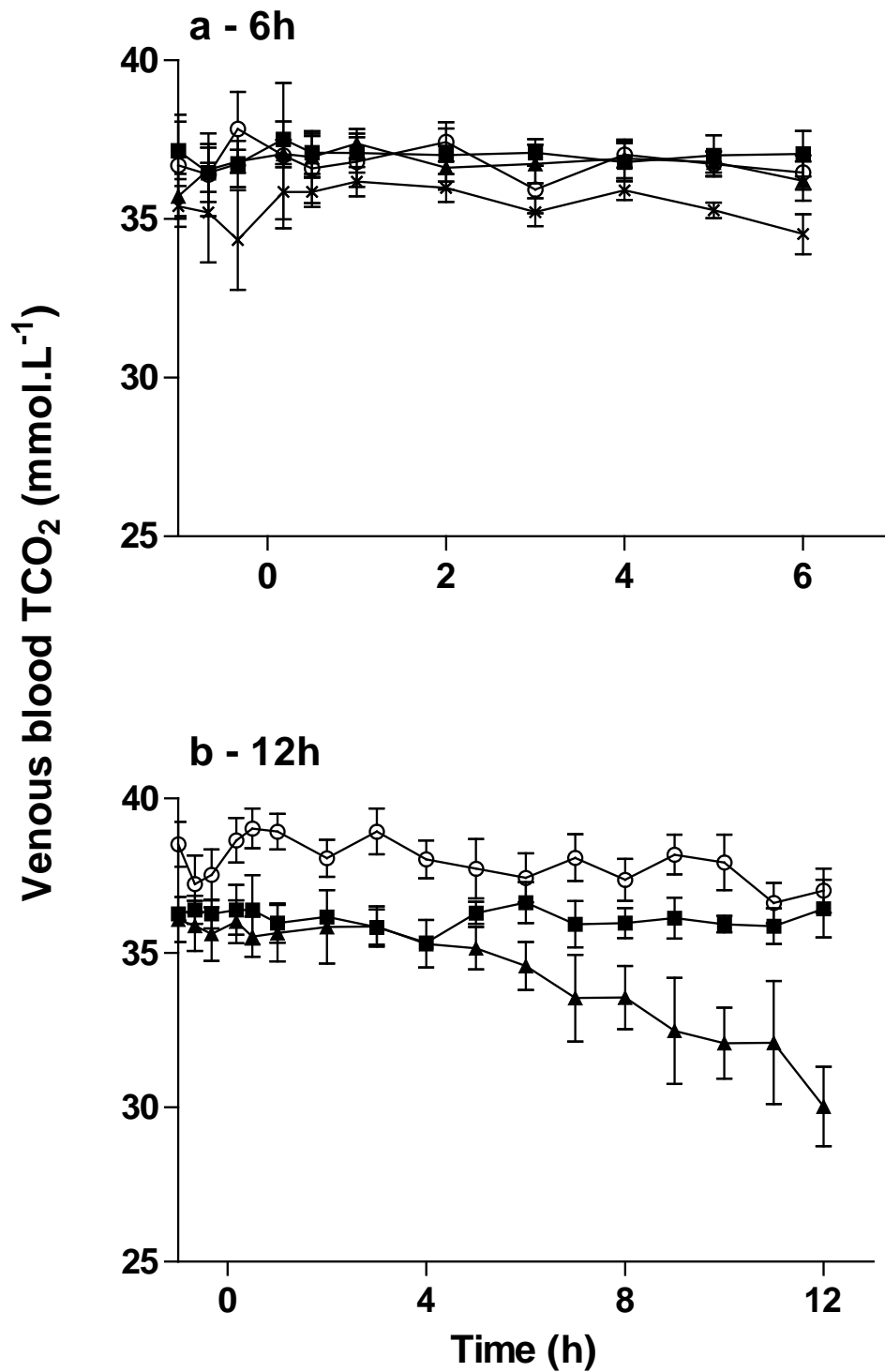
Changes in venous blood calcium (pH corrected to 7.4) following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



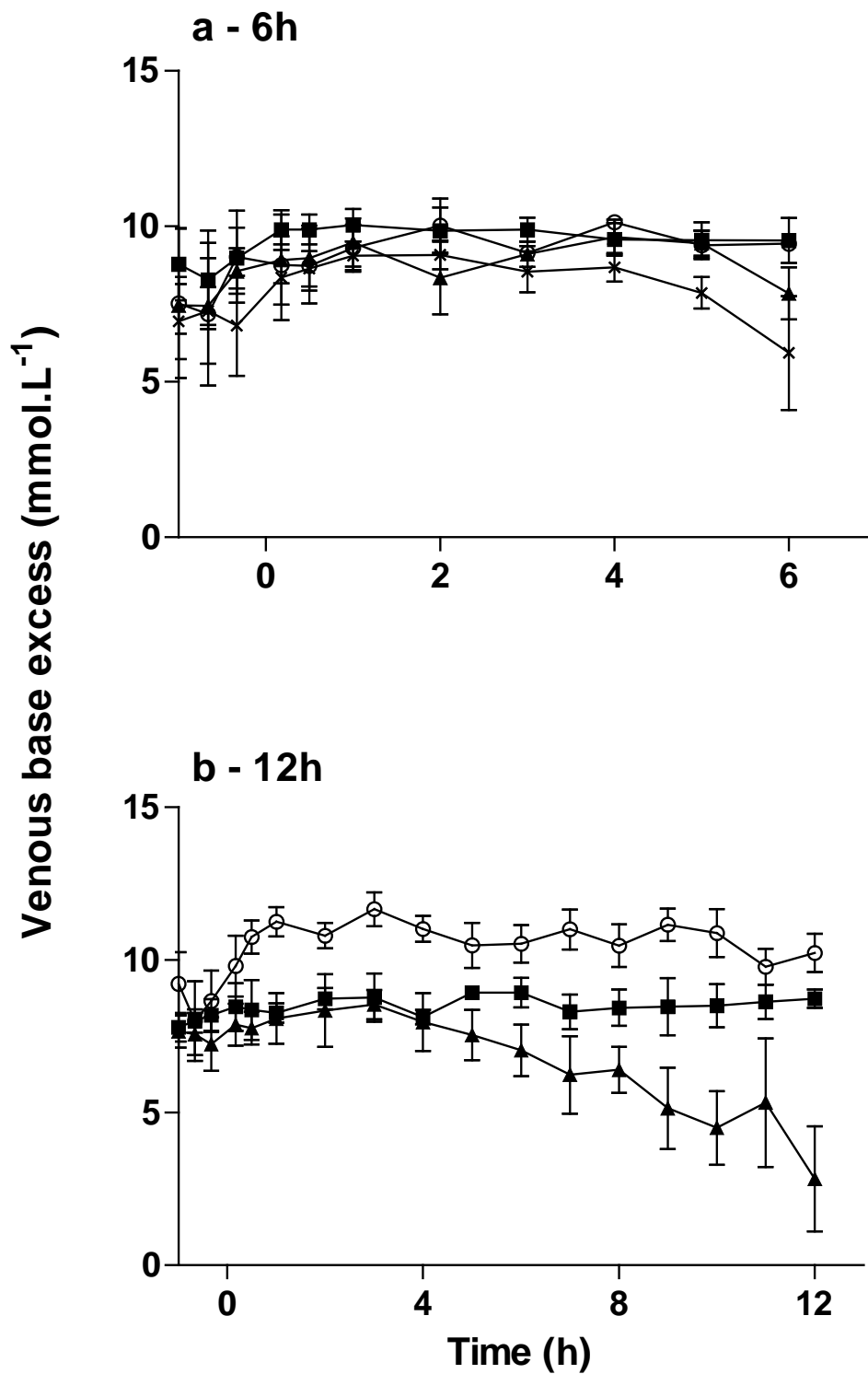
Changes in venous blood bicarbonate following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (○). Data are expressed as mean \pm SE.



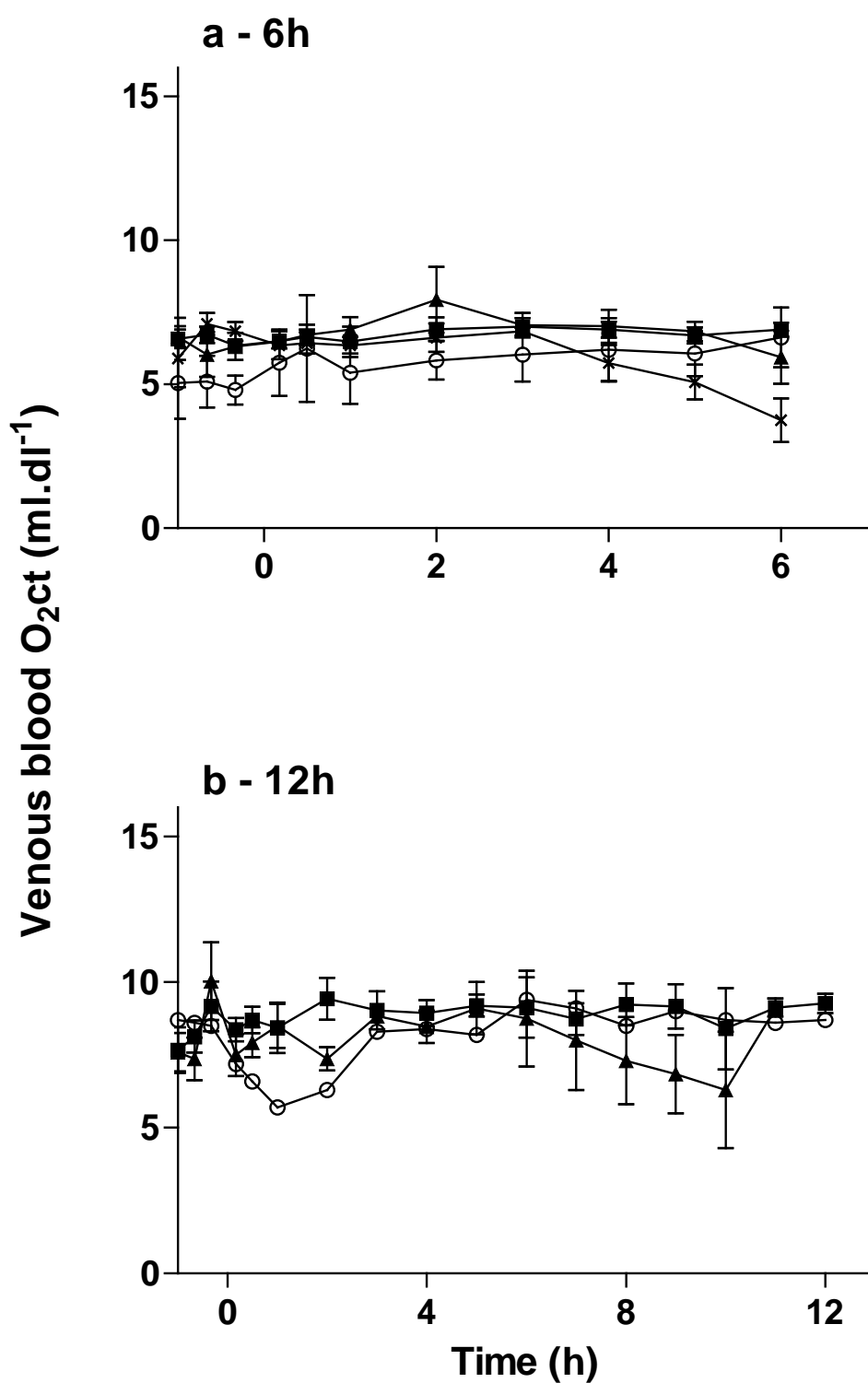
Changes in venous blood bicarbonate (standardized) following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



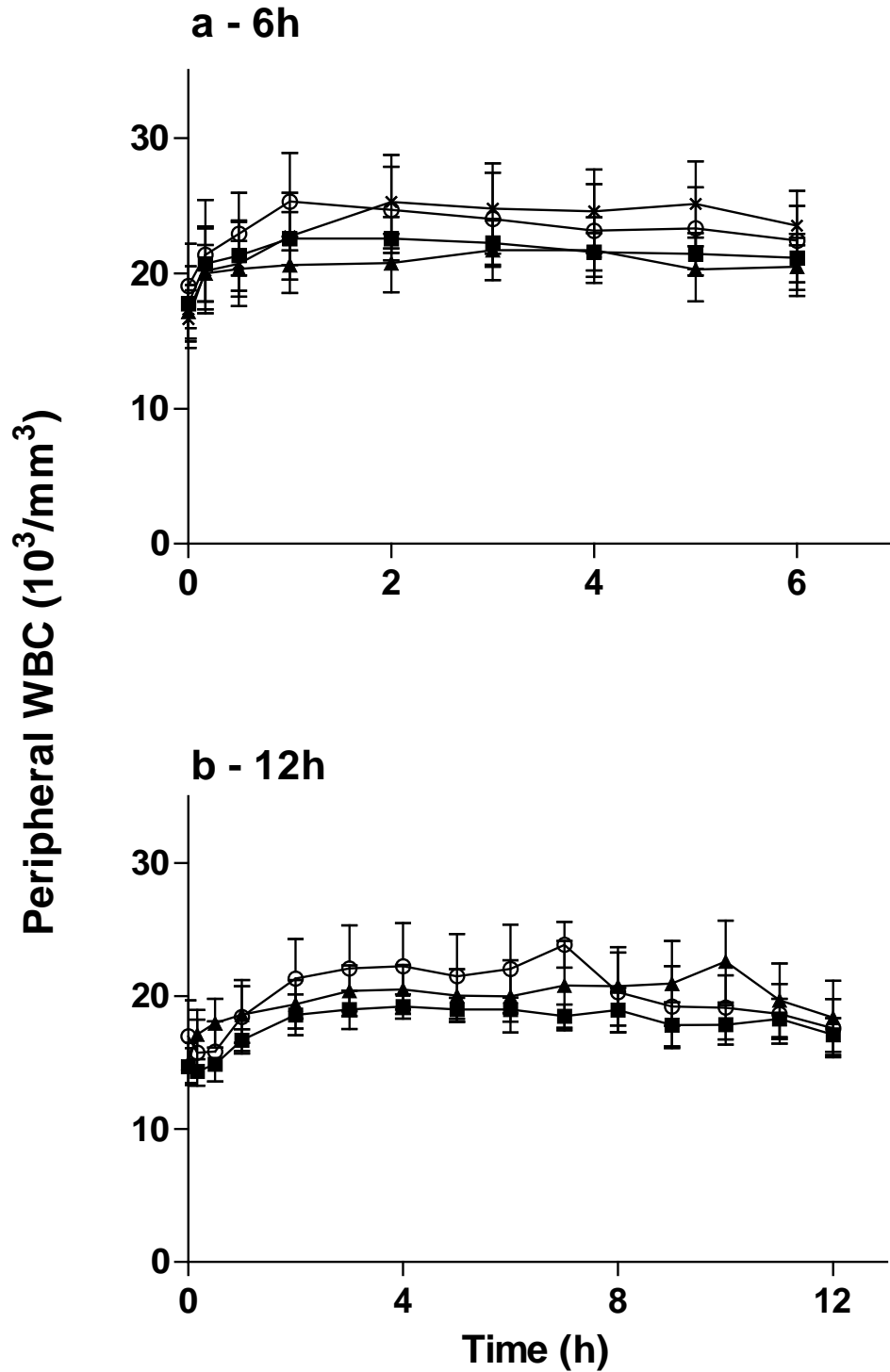
Changes in venous blood TCO₂ following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean ± SE.



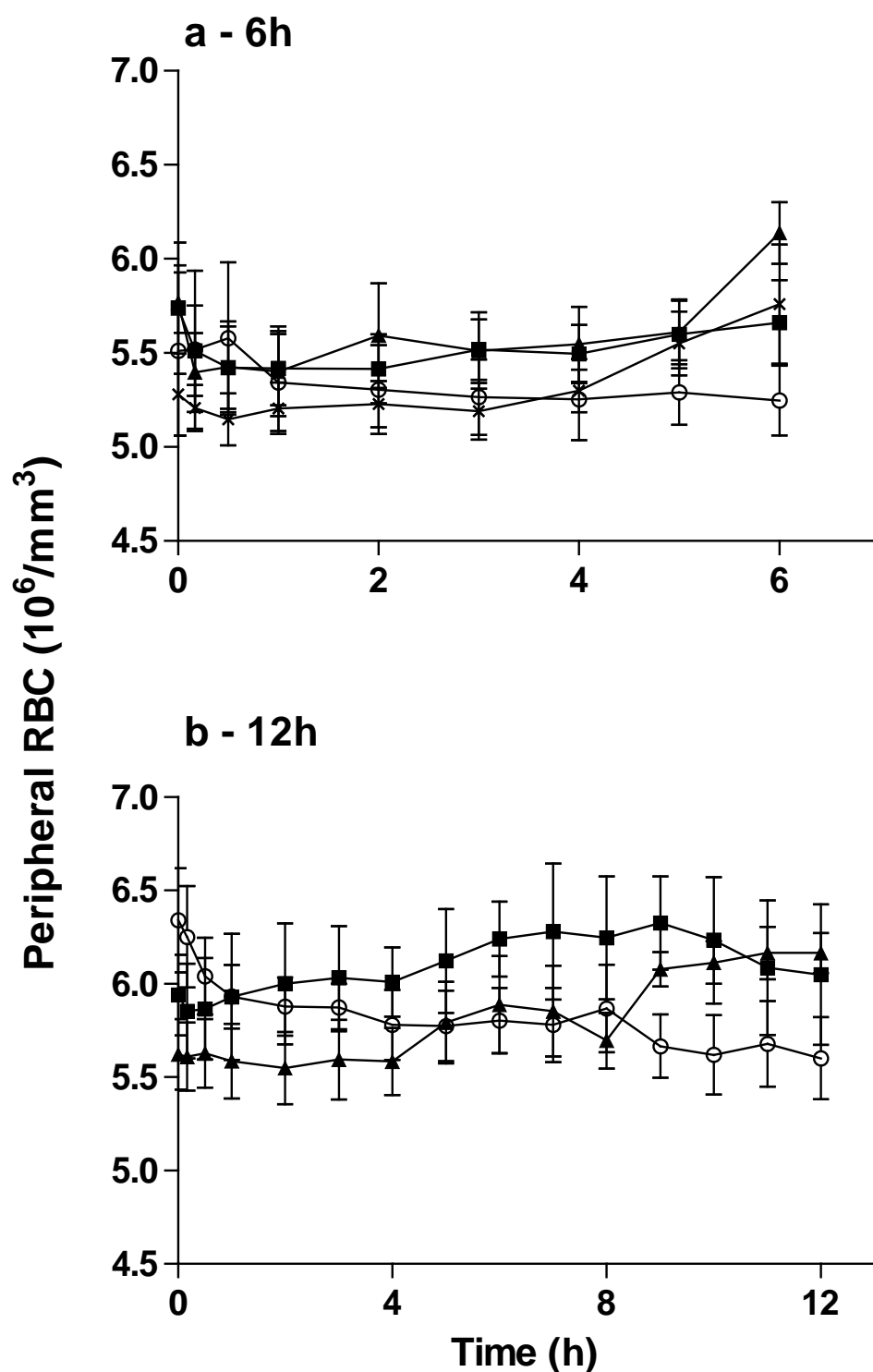
Changes in venous blood base excess following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



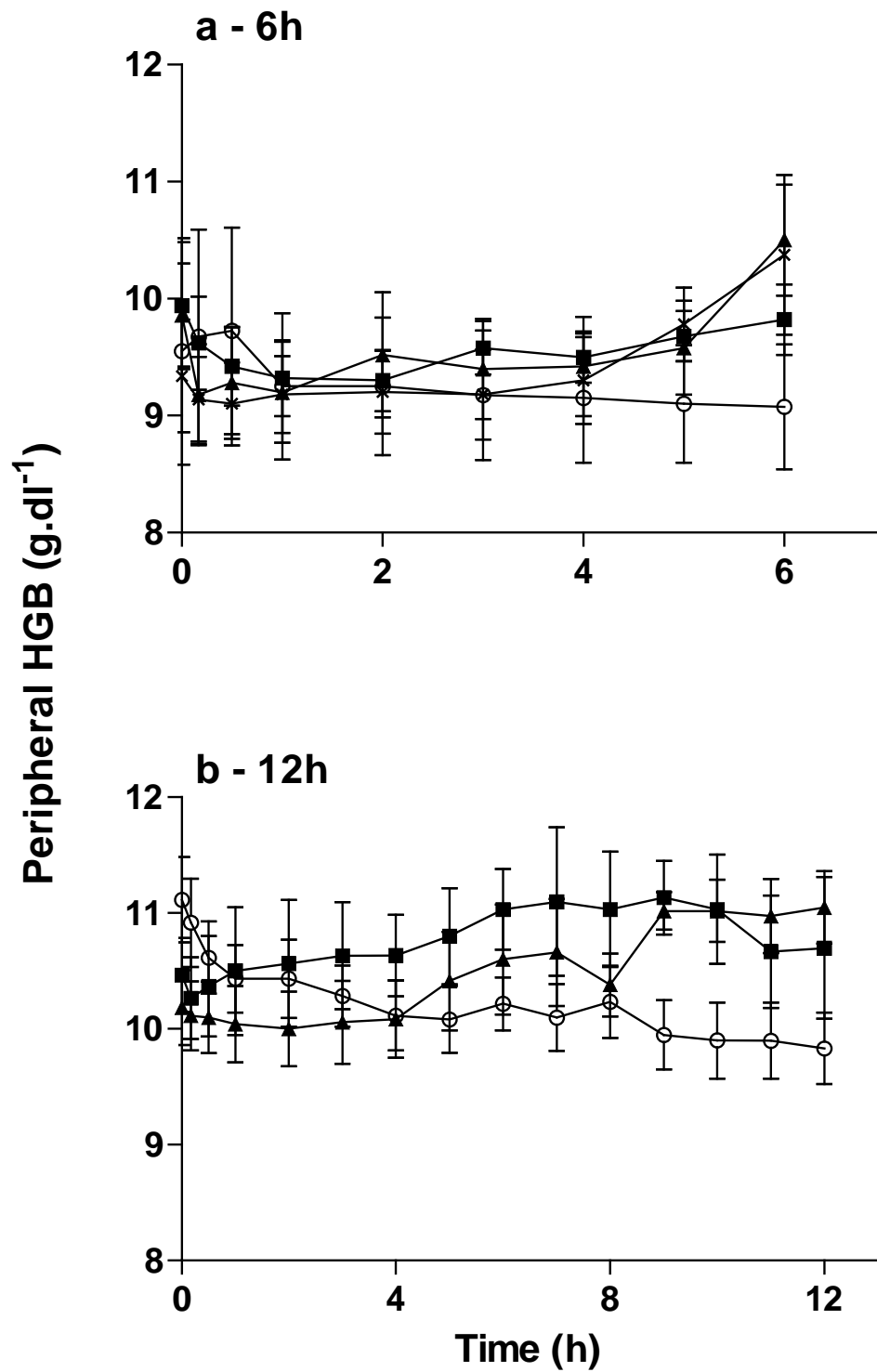
Hematology



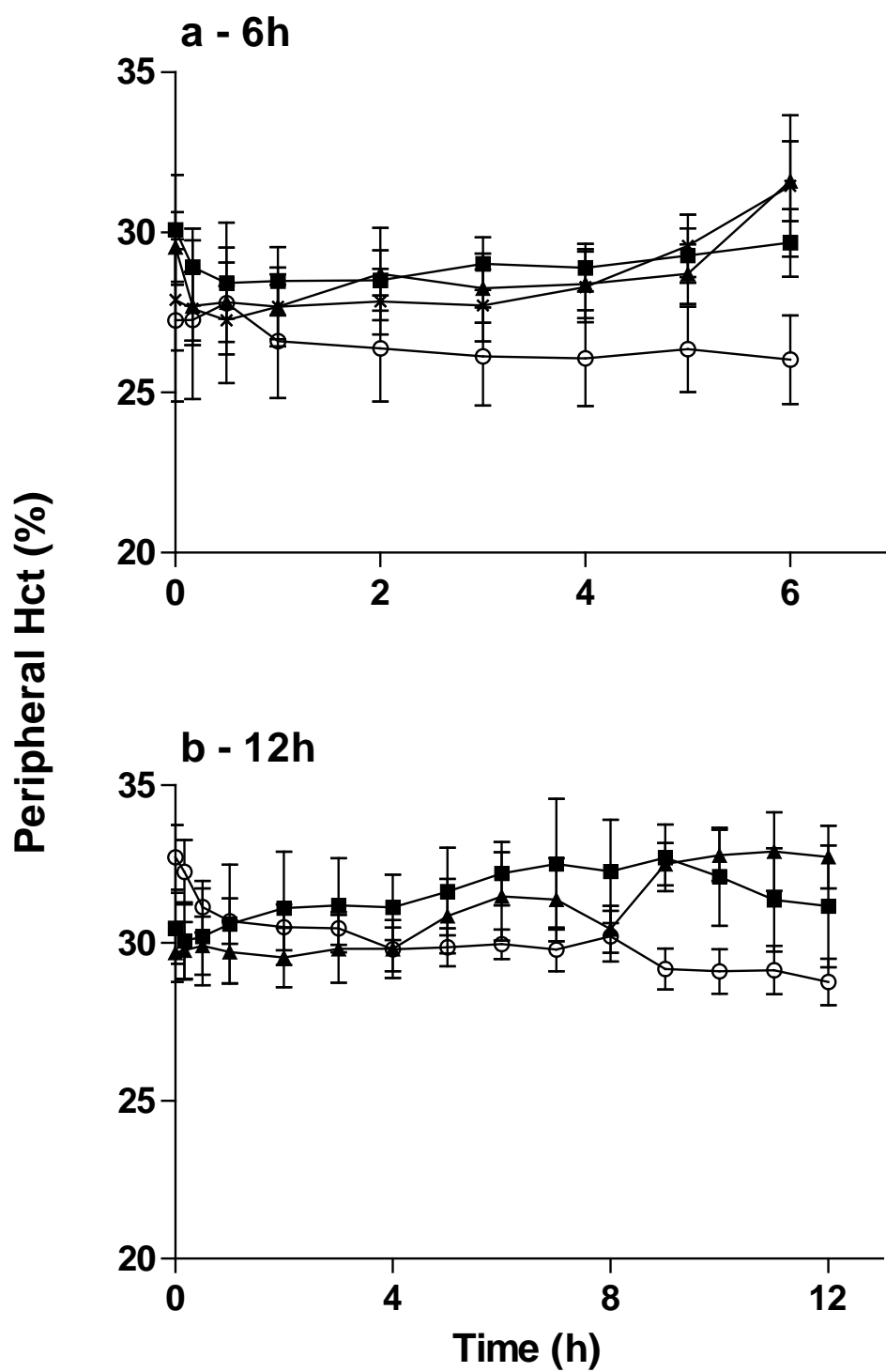
Changes in peripheral white blood cell counts following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



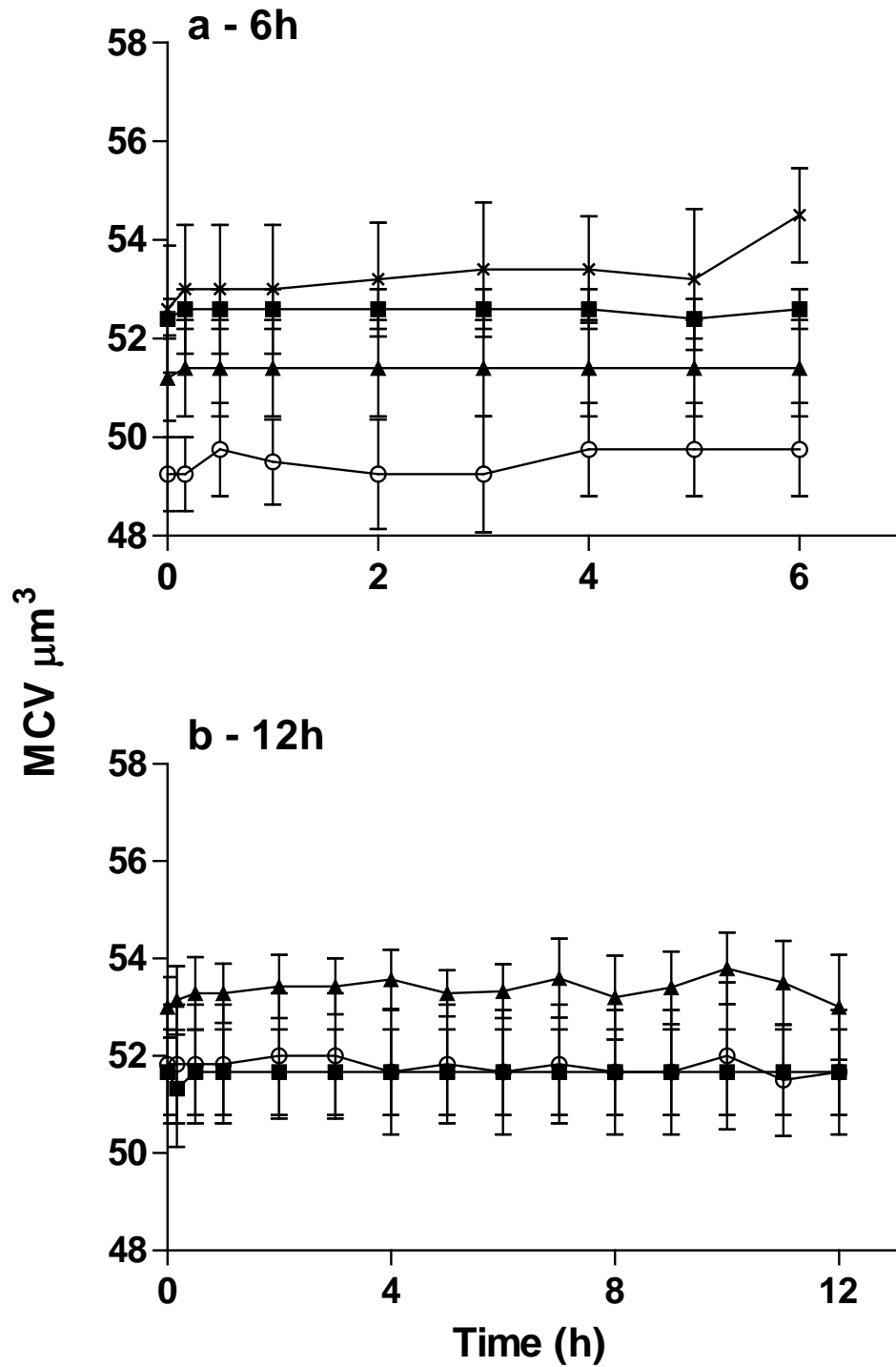
Changes in peripheral red blood cell counts following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



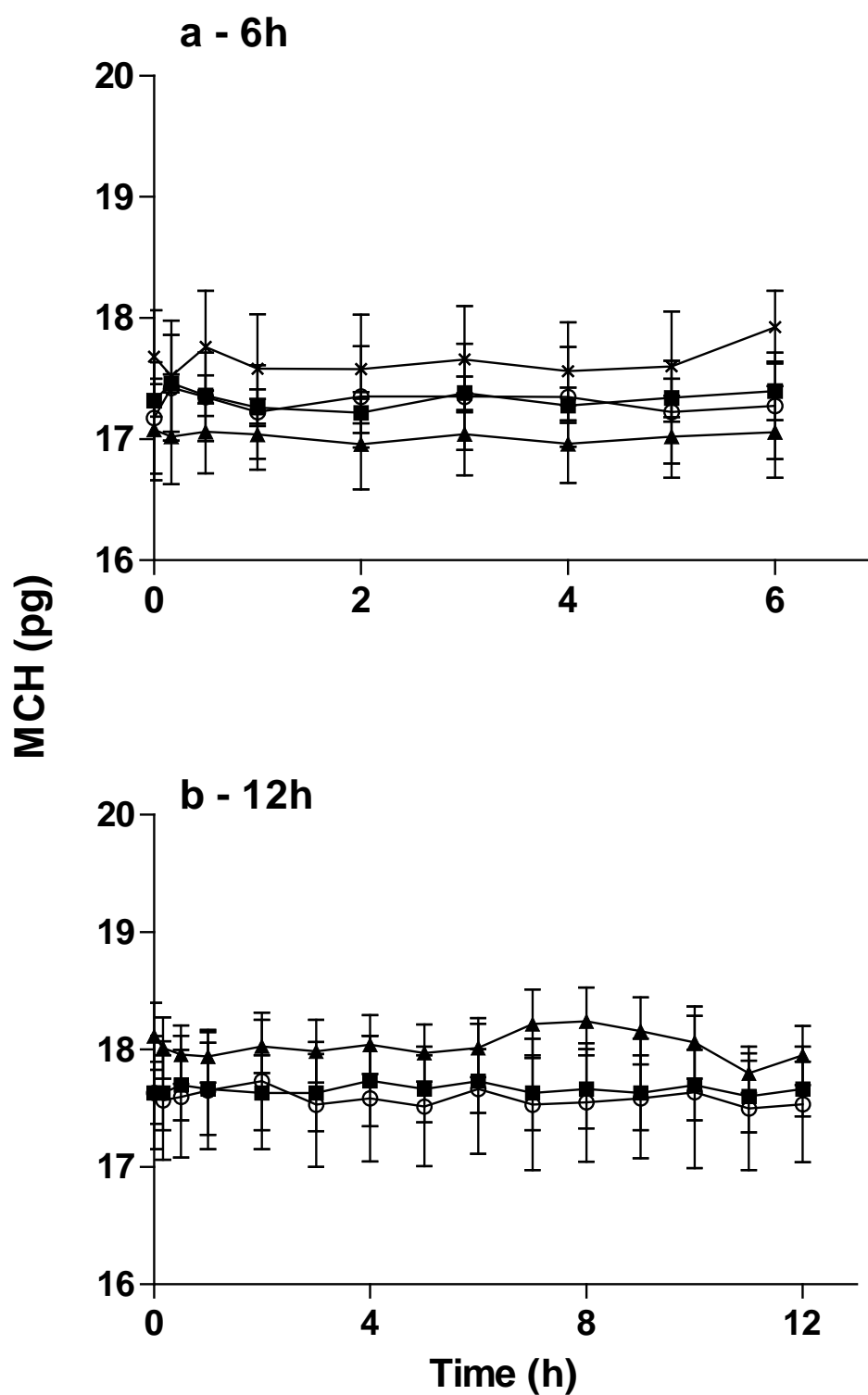
Changes in peripheral HGB following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



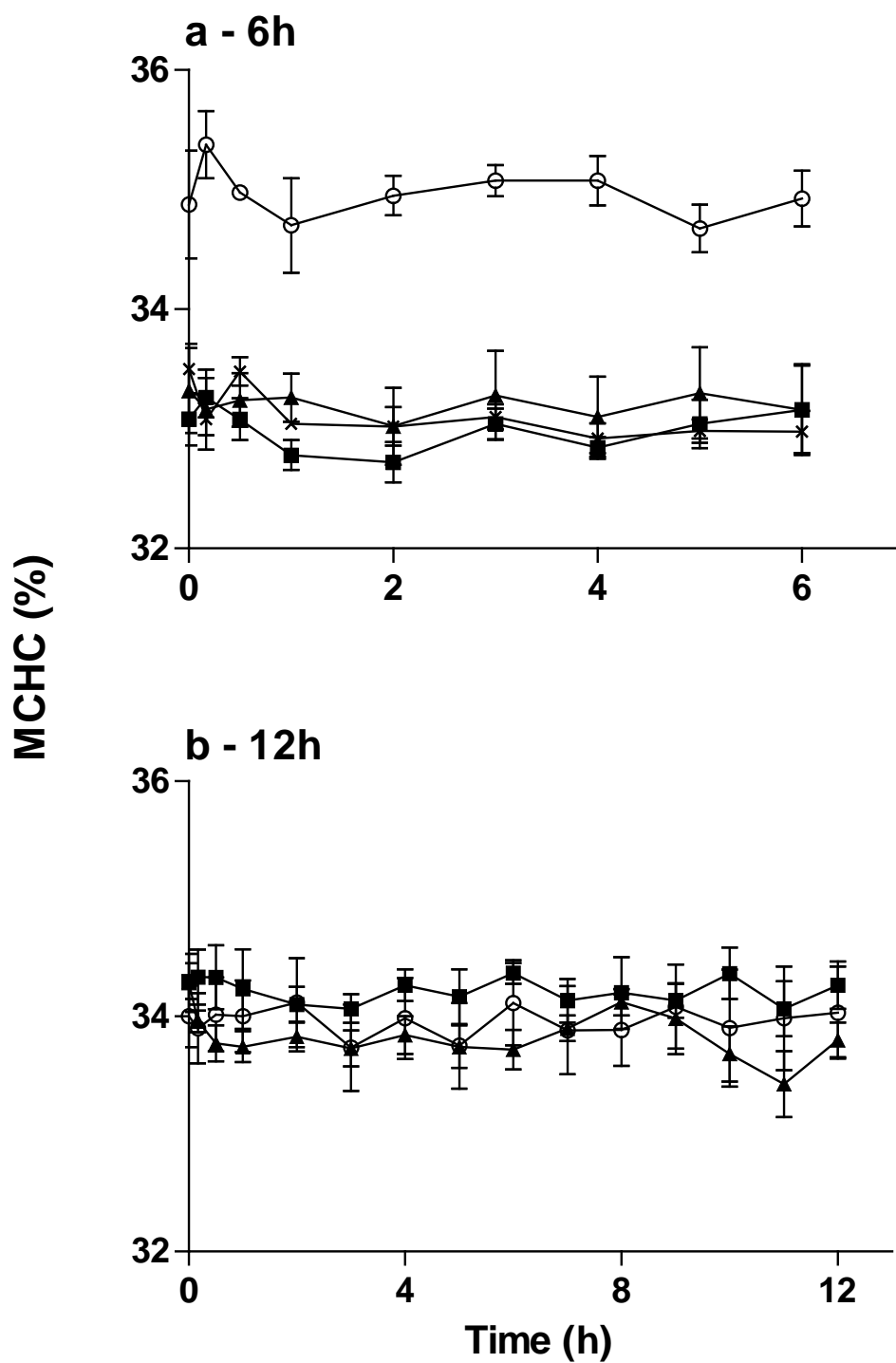
Changes in peripheral hematocrit following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (○). Data are expressed as mean \pm SE.



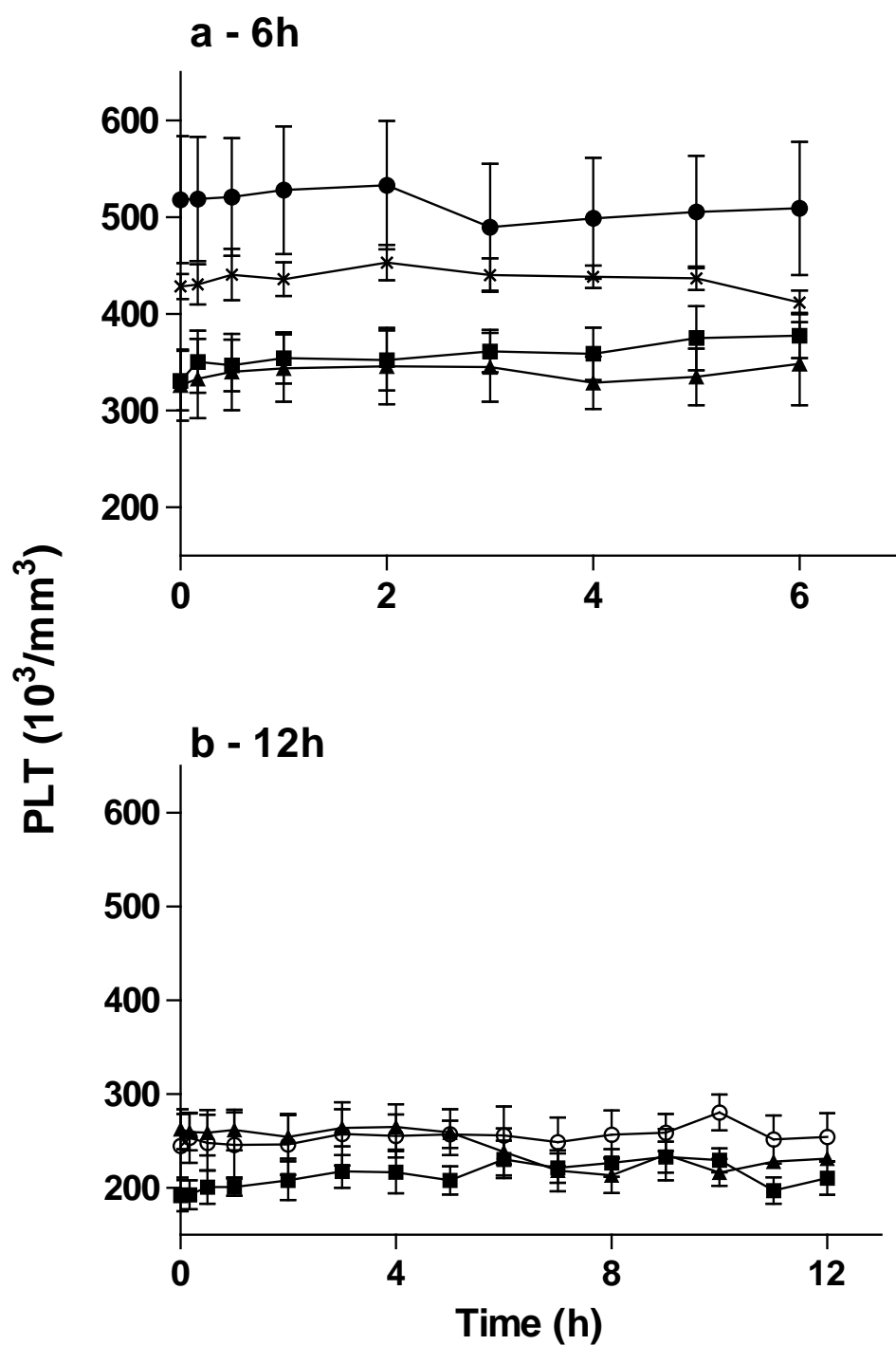
Changes in peripheral MCV following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



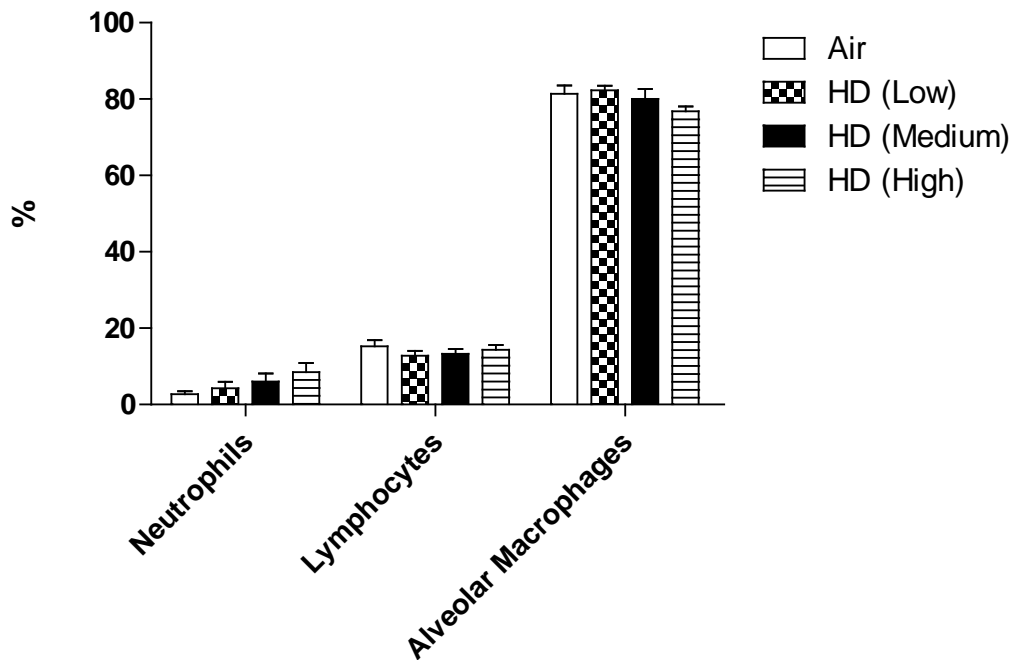
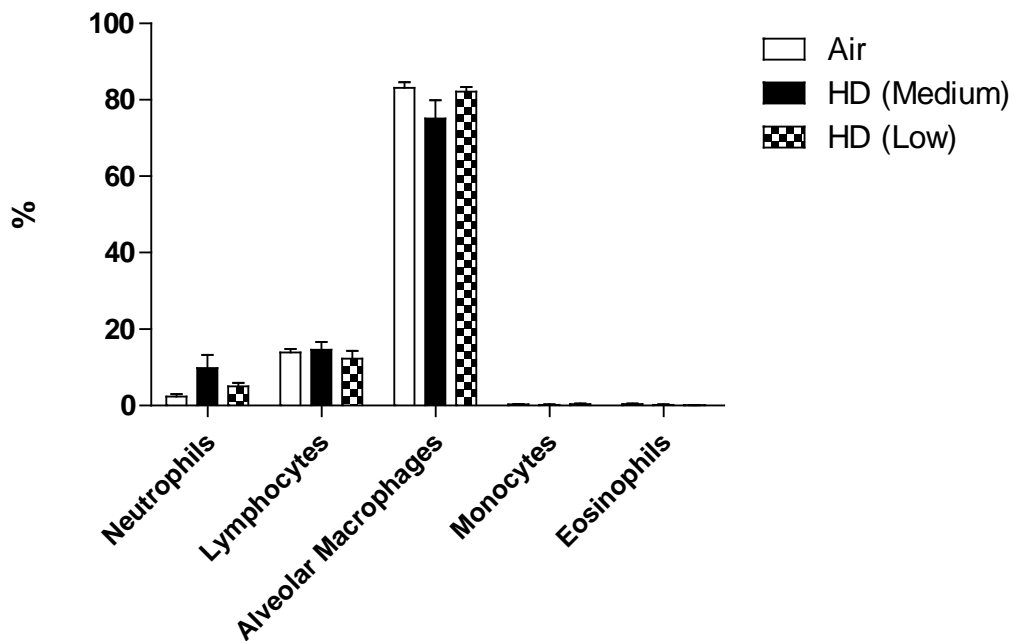
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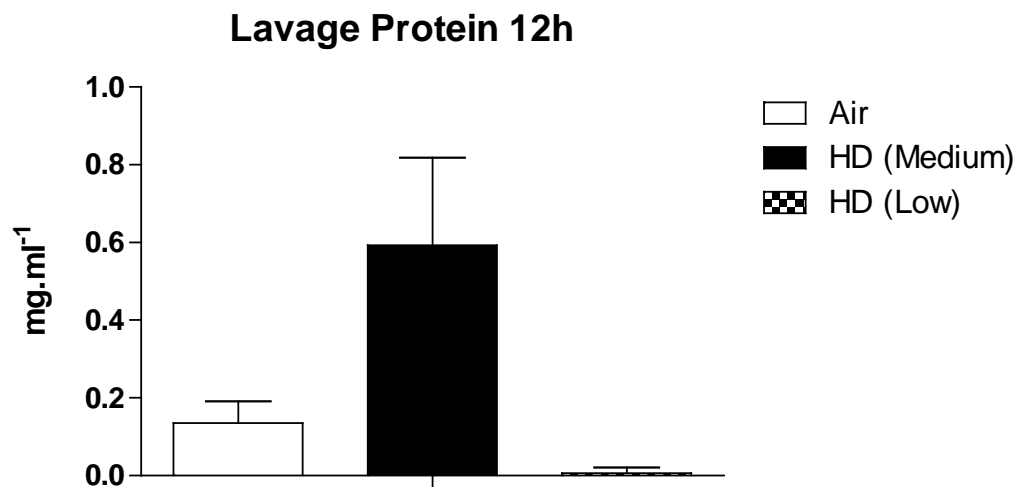
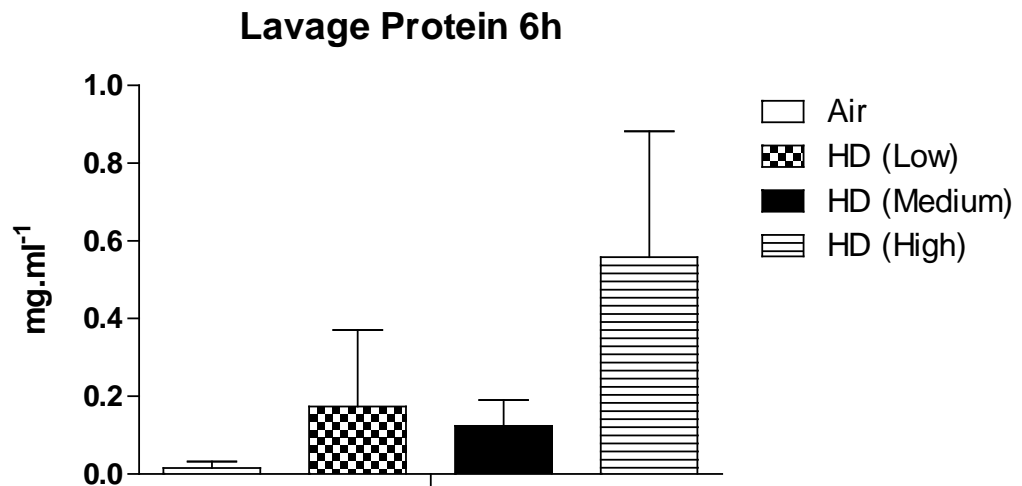
Changes in peripheral MCHC following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.



Changes in peripheral platelets following exposure to low (■), medium (▲) or high (x) doses of HD, or exposure to air (o). Data are expressed as mean \pm SE.

Differential WBC Count (1 in 5 Lavage) 6h**Differential WBC Count (1 in 5 Lavage) 12h**

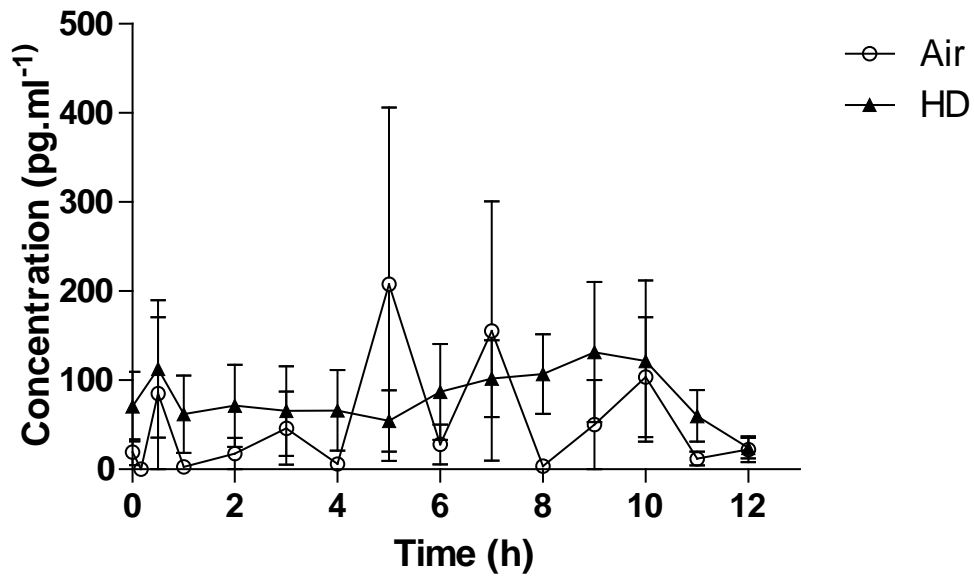
Changes in differential white blood cell counts in bronchoalveolar lavage (diluted 1 in 5 with saline) following exposure to low, medium or high doses of HD, or exposure to air. Data are expressed as mean \pm SE.



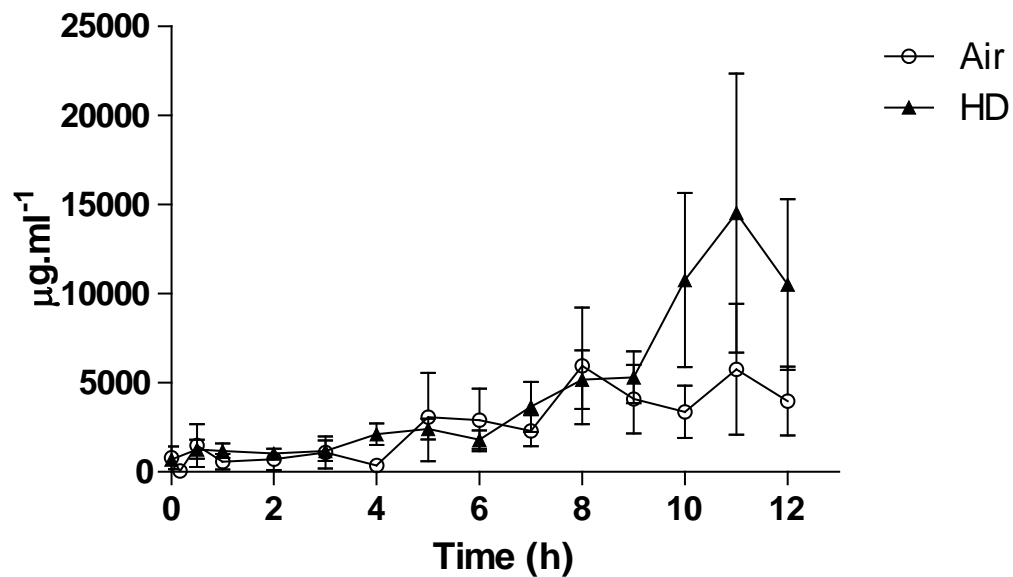
Changes in lung protein measured in bronchoalveolar lavage (diluted 1 in 5 with saline) following exposure to low, medium or high doses of HD, or exposure to air. Data are expressed as mean \pm SE.

Inflammatory Mediators

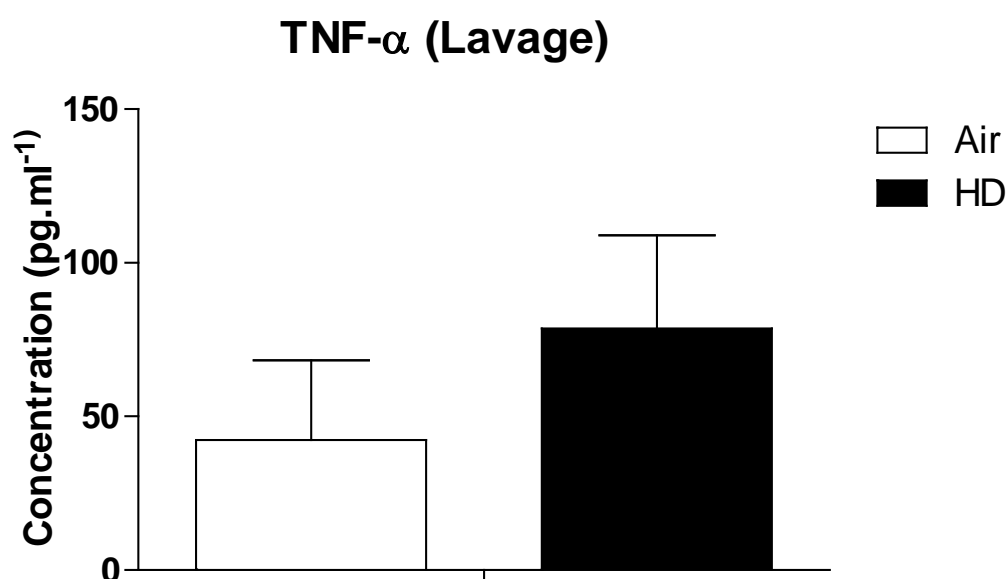
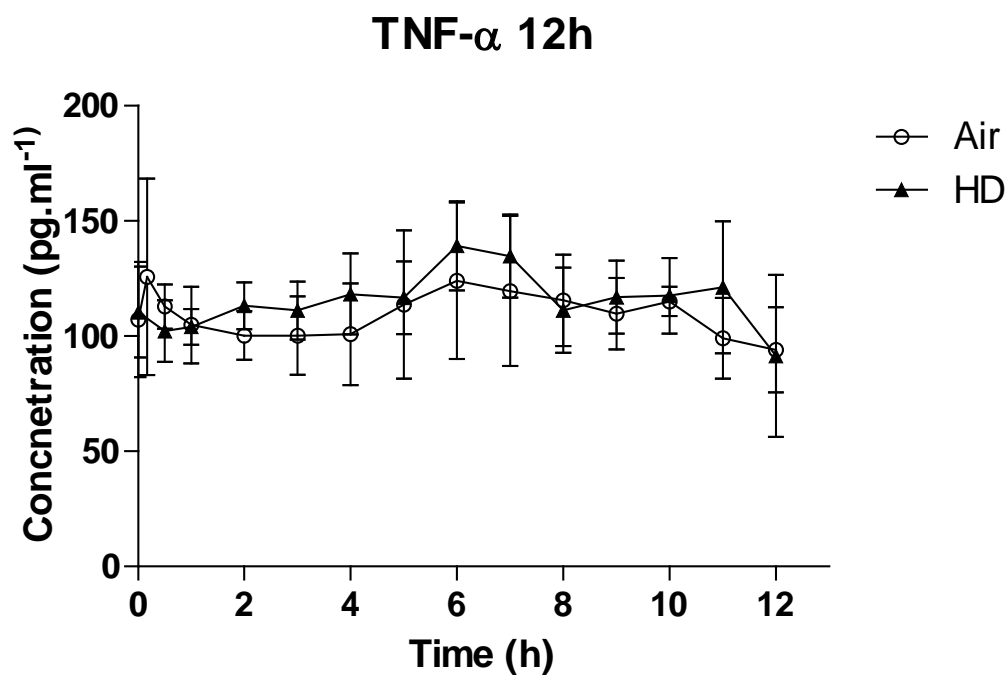
IL-6 12h



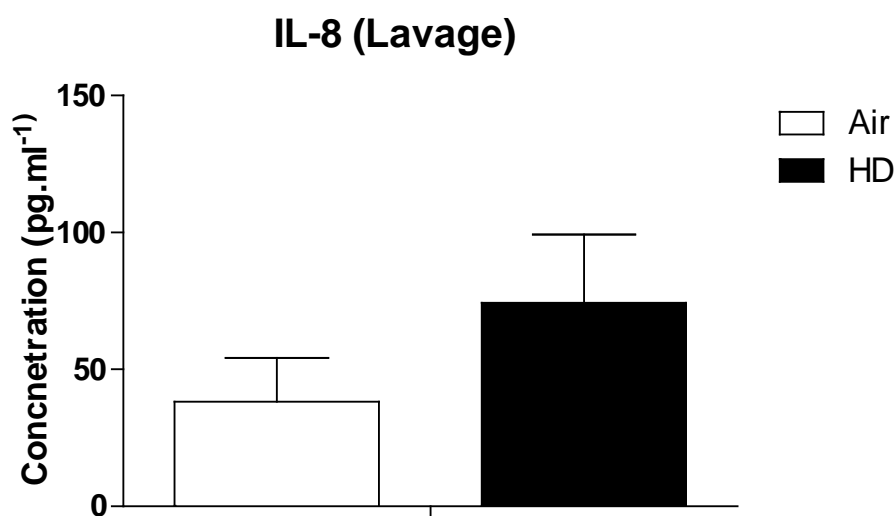
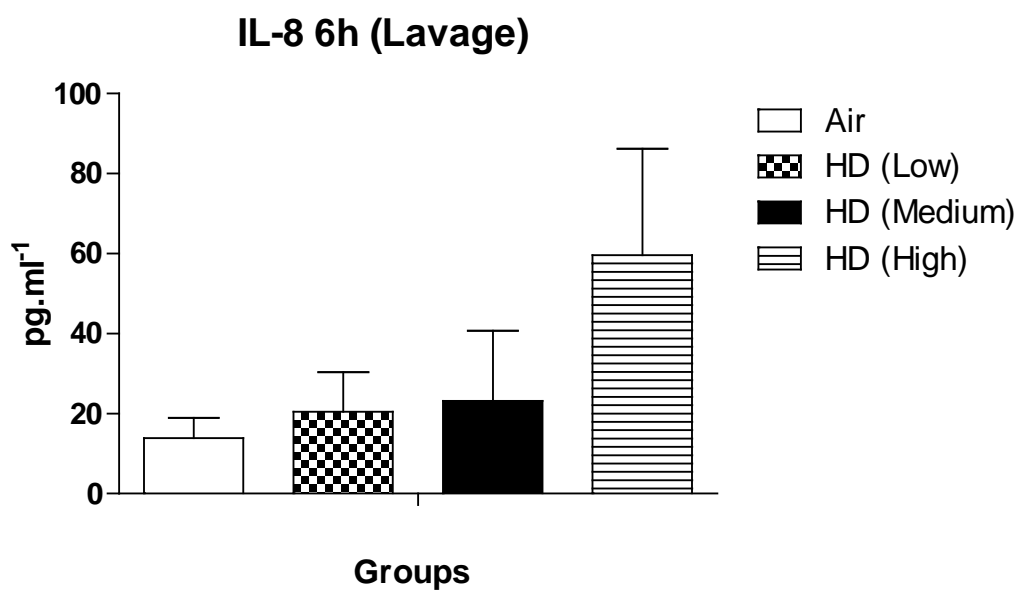
CRP 12h



Changes in peripheral IL-6 and CRP following exposure to medium (▲) doses of HD, or exposure to air (○). Data are expressed as mean ± SE



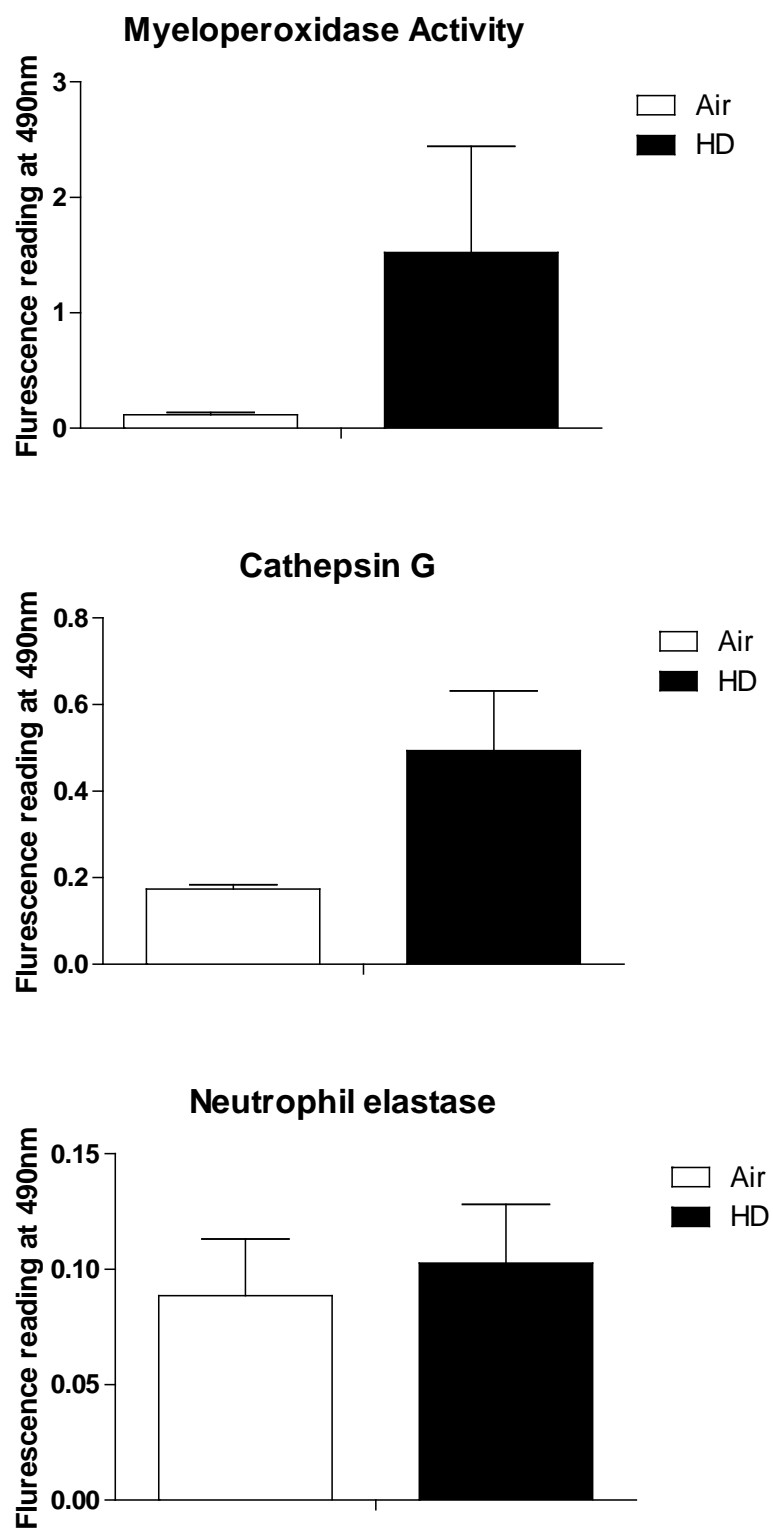
Changes in TNF- α (measured in plasma and bronchoalveolar lavage) following exposure to medium (▲) doses of HD, or exposure to air (○). Data are expressed as mean \pm SE



Changes in IL-8 measured in bronchoalveolar lavage following exposure to low, medium or high doses of HD, or exposure to air (o). Data are expressed as mean \pm SE

Portsmouth University 12h Study Analysis

Results previously obtained as proof of principle work with limited samples air (n=2) and HD (n=3). Annex A has the full study report and analysis.



Initial distribution

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Sulfur mustard (HD) is best known as a blister agent but the majority of deaths are associated with pulmonary damage. Inhalation of HD vapor can cause life threatening lung injury for which there is currently no specific medical therapy, available treatment remaining largely supportive. This treatment often requires intensive care facilities which may become overwhelmed in mass casualty events, and may be of limited benefit in severe cases. There therefore remains a need for evidence based treatment strategies to inform both military and civilian medical response teams on the most appropriate treatment for chemically induced lung injury. A clinically relevant *in vivo* large animal model has been established and a dose ranging study performed to inform the 12 hour toxicology study. This study has demonstrated changes in oxygenation and acid/base balance in HD exposed animals as well as pathology consistent with anecdotal human poisoning. This model will be used to investigate novel therapies targeting HD-induced lung injury.

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ORIGINAL ARTICLE

Exposure–response effects of inhaled sulfur mustard in a large porcine model: a 6-h study

S.J. Fairhall¹, B.J.A. Jugg¹, R.W. Read¹, S.J. Stubbs¹, S.J. Rutter¹, A.J. Smith¹, T.M. Mann¹, J. Jenner¹, and A.M. Sciuto²

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Abstract

Context: Inhalation of sulfur mustard (HD) vapor can cause life-threatening lung injury for which there is no specific treatment. A reproducible, characterized *in vivo* model is required to investigate novel therapies targeting HD-induced lung injury.

Materials and methods: Anesthetized, spontaneously breathing large white pigs (~50 kg) were exposed directly to the lung to HD vapor at 60, 100, or 150 µg/kg, or to air, for ~10 min, and monitored for 6 h. Cardiovascular and respiratory parameters were recorded. Blood and bronchoalveolar lavage fluid (BALF) were collected to allow blood gas analysis, hematology, and to assay for lung inflammatory cells and mediators. Urine was collected and analyzed for HD metabolites. Histopathology samples were taken postmortem (PM).

Results: Air-exposed animals maintained normal lung physiology whilst lying supine and spontaneously breathing. There was a statistically significant increase in shunt fraction across all three HD-exposed groups when compared with air controls at 3–6 h post-exposure. Animals were increasingly hypoxemic with respiratory acidosis. The monosulfoxide β-lyase metabolite of HD (1-methylsulfinyl-2-[2(methylthio)ethylsulfonyl]ethane), MSMTSE, was detected in urine from 2 h post-exposure. Pathological examination revealed necrosis and erosion of the tracheal epithelium in medium and high HD-exposed groups.

Conclusion: These findings are consistent with those seen in the early stages of acute lung injury (ALI).

Keywords: Acute lung injury; histopathology; inflammation; inhalation; monosulfoxide; physiology; porcine; sulfur mustard (HD)

Introduction

The use of sulfur mustard (bis(2-chloroethyl)sulfide; HD) as a chemical warfare (CW) agent remains of concern to the Armed Forces. Since its first use in World War I (Prentiss, 1937), HD has been used in numerous locations across the world by aggressors (Stockholm International Peace Research Institute [SIPRI], 1971). This includes during the Iran–Iraq conflict in the 1980s where the use of HD resulted in over 100,000 medical casualties (Pechura and Rall, 1993; Hefazi et al., 2005).

Though primarily a dermal vesicant, it is the inhaled effects of HD, which account for the majority of deaths following HD poisoning. Indeed, the majority of deaths from HD poisoning during World War I had some degree of pulmonary involvement (Prentiss, 1937). The respiratory effects

following exposure to HD vapor range from mild effects such as an unproductive cough to bronchitis, necrosis of the respiratory epithelium, bronchopneumonia, pulmonary edema, and respiratory failure; often a consequence of complications such as infection (Kehe and Szinicz, 2005; Russell et al., 2006).

HD is a successful CW agent because of its low threshold of effect, causing incapacitation at a concentration of ~6.5 mg/m³ following a 60-min exposure (Prentiss, 1937). In addition, it also has a high odor detection threshold (0.6 mg/m³), making it difficult to ascertain whether one has been exposed (Pechura and Rall, 1993). Together, these properties, coupled with the persistency of HD and the delay in the onset of symptoms of ~4 h, make finding an effective therapy for HD poisoning of utmost importance. To date, no specific treatment for HD

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poisoning exists. A reliable and reproducible *in vivo* model of HD lung injury needs to be established in which potentially effective treatments could then be evaluated.

Although the effects of HD have been studied *in vitro*, the biochemical, physiological, and toxicological acute effects of HD lung injury are not fully understood. A small number of studies have been carried out to investigate HD-induced lung injury in whole animal models. These found that exposure to HD vapor resulted in the formation of pseudomembranes covering interior surfaces of the tracheobronchial tree. HD is readily absorbed within the respiratory tract and the estimated LC_{50} in mice was reported to be 101 mg·min/m³ for a 10-min exposure (Winternitz and Finney, 1920; Cameron et al., 1946; Box and Cullumbine, 1947). However, as rodents are obligate nasal breathers it is difficult to extrapolate the results to man, who is not. HD injury in rodents is subsequently more severe within the upper respiratory tract (URT), whereas in humans it is the lower respiratory tract (LRT) that is most affected. It is therefore essential to find a species in which the lung physiology is more comparable with man so that any data derived can be extrapolated with greater confidence.

This laboratory has established a large white pig model of HD-induced lung injury. The large white female pig was chosen for this study as it is a well-studied and understood species. Its lung physiology is comparable with that of man (Getty, 1975; Cramer et al., 1994) and its size means that conventional human medical equipment can be utilized due to similarities in the tracheobronchial and vascular architectures. The pig has been widely used as a model of acute lung injury (ALI) caused by inhaled agents such as chlorine (Gunnarsson et al., 1998) and phosgene (CG) (Brown et al., 2002, 2006), by bacterial infection (Dehring et al., 1983) and by physical trauma (Thörne et al., 1989; Garner et al., 2009). The use of a large animal model such as the pig also allows more extensive monitoring to be conducted with increased sampling frequency (and volume). Furthermore, bypassing the URT mimics exposure in man during periods of stress and exercise (e.g. military personnel on the battlefield or civilians running to escape a scene of chemical release), when man breathes more via the mouth than the nose, increasing the load in the deep lung. Delivery direct to the lung avoids the need to account for differences, for example, in surface area and nasal physiology, between the pig and man. Finally, our laboratory has established expertise in using this animal model (Brown et al., 2002, 2006).

A novel exposure system for the delivery of HD vapor directly to the lung has previously been reported (Fairhall et al., 2008) and preliminary respiratory data presented.

The aim of the work described here was to expose the anesthetized, spontaneously breathing large white pig to a range of doses of HD vapor directly to the lung in a reliable and quantitatively reproducible manner. The effects of exposure on lung physiology, hematology, inflammatory markers, and pathology were investigated. This study will facilitate the future development of therapeutic strategies against HD-induced lung injury.

Methods

Animals and surgical preparation

All animal work was carried out in accordance with the UK Animals (Scientific Procedures) Act 1986, and was approved by Ethical Review at Dstl, Porton Down, UK.

Large white female pigs (47–55 kg, mean weight 51.1 ± 0.55 kg) ($n=19$) were obtained from a commercial source. The animals were housed individually in straw lined pens, and were allowed food (standard pig diet) and water *ad libitum* for 5 days prior to the experiment. From 12 h prior to the experiment, access to food was withdrawn though water remained freely available.

Surgical procedure

Animals were premedicated with midazolam hydrochloride ("Hypnovel", Roche Products Ltd., Welwyn Garden City, UK) by intramuscular injection (0.6 mg/kg), and anesthesia induced by inhalation of 5% isoflurane ("IsoFlo", Abbott Laboratories, Maidenhead, UK) in 70% oxygen and 30% nitrous oxide. The animals were intubated with a size 10 endotracheal (ET) tube that had been lined with polytetrafluoroethylene (PTFE) as previously described (Fairhall et al., 2008). Surgery was performed under aseptic conditions by cut down procedure for insertion of arterial and venous catheters. A "Pulse Contour Continuous Cardiac Output" (PiCCO) catheter was inserted into the right femoral artery and attached to a PiCCO monitor, allowing continuous measurement of such parameters as cardiac output (CO), systemic artery pressure, stroke volume, and core temperature. Periodic thermal dilution analysis allowed estimation of extravascular lung water. Other noninvasive physiological monitoring devices were attached to a Propaq 106EL monitor. In the pig, the presence of a suburethral diverticulum makes urethral catheterization difficult and, therefore, a Bonanno catheter was introduced into the bladder via an open cystotomy.

Throughout the study, a maintenance infusion of 0.9% w/v sodium chloride and 4% w/v glucose (2.5 mL/kg/h) was delivered to replace insensible losses.

Once surgery was completed, intravenous anesthesia was established and maintained using fresenius propoven 1% (10–12 mg/kg/h) (Propofol® Fresenius Kabi Ltd., Cheshire, UK) and alfentanil hydrochloride (0.5–2.5 µg/kg/h) (Rapifen® Janssen-Cilag Ltd., High Wycombe, UK).

Exposure to HD

The system for exposing the animals to HD has been described in detail previously (Fairhall et al., 2008). In brief, the animals were maintained supine whilst breathing spontaneously and baseline measurements were taken for 1 h. They were then connected to the exposure apparatus via a PTFE-lined ET tube, a size 2 Fleisch pneumotachograph and a sample port for 10 min. HD vapor was generated from two custom "Porton" vapor generators (PVGs) operated in parallel. The apparatus was set up to deliver the required inhaled dose over a 10-min exposure. Animals were randomly

assigned to either air control ($n=4$), 60 $\mu\text{g/kg}$ HD ($n=5$), 100 $\mu\text{g/kg}$ HD ($n=5$), or 150 $\mu\text{g/kg}$ HD ($n=5$) groups.

HD concentration was measured in real time using a Total Volatile Organic Compound photoionization detector (TVOC PID) (Ion science, Royston, UK) and by sampling the vapor during exposure at a constant rate onto Porapak adsorption tubes. These were then analyzed by solvent (hexane) elution and quantitative gas chromatography–flame photometric detection (GC-FPD). For this, a mid-polar capillary column (DB1701 15 m \times 0.32 mm, 1 μm film; Agilent Technologies, Stockport, UK) was used with helium carrier gas at a flow rate of 2.5 mL/min.

Physiological measurements

Cardiac and pulmonary physiological parameters were recorded every 5 min for the first 30 min following exposure and then every 30 min throughout the course of the experiment (up to 6 h). Derived variables were calculated using standard formulae proposed by Edwards et al. (1993).

Arterial and mixed venous blood samples were taken prior to exposure, immediately post-exposure, at 30 min post-exposure and then hourly post-exposure for the duration of the experiment. An aliquot of arterial blood was placed into a heparinized blood gas syringe (Sims Portex Ltd., UK) and immediately analyzed using a GEM Premier 3000 blood gas analyzer (Instrumentation Laboratories UK Ltd., Cheshire, UK). A second aliquot of arterial blood was mixed and refrigerated for later hematological analysis. A third sample was separated into appropriate anticoagulants, centrifuged (3000 rpm, 10 min), and the plasma stored at -80°C for analysis. In addition to the collection of blood, total urine output was noted hourly and 12-mL aliquot samples retained (-80°C).

Respiratory parameters were recorded in real time using eDAcq (EMMS Data Acquisition) software (Infodisp, UK). Minute and tidal volume, lung resistance and compliance, along with other lung function variables, were monitored in real time throughout, and subsequent to, the exposure. For the exposures, a specific inhaled dose was targeted for each of the three HD groups. This was calculated using concentration, time, the animal's body weight, and total inhaled volume (inhaled dose ($\mu\text{g/kg}$) = concentration ($\mu\text{g/L}$) \times (volume breathed per min (L/min) \times exposure time (min)) / body weight (kg)).

The concentration of HD generated for each group was kept constant and the desired inhaled dose reached by varying the length of exposure. Concentration was kept constant as the PVGs are slow to equilibrate following a temperature change and therefore not practicable to keep changing during an exposure. Effort was also made to maintain the animals' minute volumes between 6 and 8 L/min by controlling the dose of anesthetic. The inhaled dose was then estimated every minute during the exposure, and the exposure terminated when the inhaled dose reached the desired value. Thus, whilst the exposure duration was planned to be 10 min, in practice it varied between 6.5 and 12.6 min to account for uncontrollable variations in the animal's minute volume from 7 L/min.

Postmortem and histopathology

At the end of the 6-h monitoring period, or when the animal became moribund (defined as asystole and a mixed venous oxygenation (SvO_2) of $<15\%$) the animal was culled by an intravenous overdose of sodium pentobarbitone, 200 mg/mL ("Euthatal" Rhone Merieux Ltd., Harlow, Essex) and a full postmortem (PM) examination carried out. PM was carried out wearing personal S10 respirators to prevent contamination from any potential HD "off-gassing." Following removal of the ET tube, the trachea was clamped, the thorax opened, and after the animal was exsanguinated by severing the major arteries, the lungs and heart were removed. A small incision was made in the trachea just below the clamp. A flexible bronchoscope (Fiberoptic Bronchoscope, Olympus BF-4B2) was then inserted and maneuvered into the right median lobe. Aliquots of sterile saline ($4 \times 40\text{ mL}$) were expelled into the lobe, the syringe pulled back, and the fluid aspirated between each aliquot. The aliquots were pooled together and placed on ice. Following lavage, the lungs were weighed for lung wet weight/body weight (LWW:BW) ratio determinations (the weight of the remaining lavage saline being taken into account). Samples from each of the lobes, together with samples from other major organs were taken, fixed in neutral buffered formalin, and processed for histopathological examination using standard methods.

Sample processing and hematology

A standard eight test hematological analysis was carried out on arterial blood samples and bronchoalveolar lavage fluid (BALF) (4°C) using a Coulter Ac-T 5 diff CP (Beckman Coulter) series analyzer. Differential white blood cell (WBC) counts (macrophages, lymphocytes, monocytes, eosinophils, neutrophils, and basophils) were obtained manually from blood smears and BALF slides (Shandon Cytospin, 1:5 dilution with 0.9% w/v sodium chloride, 1800 rpm, 10 min) stained with DifQuik, using a Zeiss Axioskop 40 microscope. The remaining BALF was spun (10 min, 3000 rpm), and the supernatant used to determine protein content (Coomassie blue; Bradford, 1976). Twenty milliliters of supernatant were stored (-80°C) for future analysis of inflammatory mediators. Mediators were measured using commercially available standard quantitative porcine ELISA kits (R&D Systems, Abingdon, UK).

Urine samples were analyzed for the monosulfoxide β -lyase metabolite (1-methylsulfinyl-2-[2(methylthio)ethylsulfonyl]ethane), MSMTESE using the method published by Read and Black (2004), modified by use of an alternative C_{18} liquid chromatography (LC) column (ACE 3 C18HL, 150 mm \times 2.1 mm; Hichrom Ltd., Reading, UK). Calibration was performed using urine (from pigs exposed to air) that had been spiked with MSMTESE over the range 0.1–50 ng/mL. Deuterated (D_6) MSMTESE was used as internal standard for quantitation.

Statistical analysis

Samples were taken at three points pre-exposure. The values obtained at these time points were averaged and are

presented here as a single baseline data point ($t=0$). The data for each recorded parameter were grouped into one of two categories; 0–3 h and 3–6 h and were checked for normality using an Anderson–Darling test. Data that were not normally distributed were log-transformed where possible. The area under the curve (AUC) was then calculated for each parameter within each category. Comparisons of the AUC were then made between the control group and HD treatment groups (60 $\mu\text{g}/\text{kg}$ (low), 100 $\mu\text{g}/\text{kg}$ (medium), and 150 $\mu\text{g}/\text{kg}$ (high)) using either a two-sample t -test or, for nonparametric data, a Mann–Whitney U -test. Data were expressed as mean (\pm SEM) and P -values <0.05 were considered significant. For statistical analysis of inhaled dose, cytokine and monosulfoxide levels, cell differentials and LWV:BW ratio determinations, Bartlett's test was used to assess whether equal variance could be assumed and normality assessed using normal probability plots. Two-sample t -tests and analysis of variance (ANOVA) were then carried out. For nonparametric data, Kruskal–Wallis tests were adopted.

Results

HD exposures

All animals exposed to low- or medium-dose HD vapor survived up to 6 h. In the high-dose group, four animals survived to the end of the study with two animals dying within ~30 min of the 6-h time point.

Figure 1 shows that the target and achieved inhaled doses for each of the dosing groups were not significantly different. Achieved mean inhaled doses were 67.2 ± 4.3 , 100.6 ± 4.6 , and 157.8 ± 10.0 $\mu\text{g}/\text{kg}$ for the low, medium, and high groups, respectively ($P=0.17$, 0.90 , and 0.48 compared with target equivalent). This demonstrates that the exposure system could be successfully used to control the inhaled dose as desired. The three achieved mean inhaled dose groups were significantly different from each other ($P<0.001$).

Physiology and hematology

Air-exposed animals maintained arterial oxygenation throughout the study whilst there was a significant decrease in

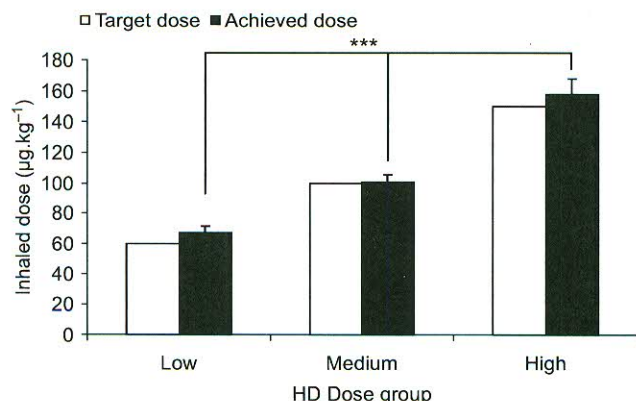


Figure 1. Target versus achieved doses for each of the HD-exposed groups ($n=5$). Data are expressed as mean \pm SE. ***Significant difference between each of the HD dose groups ($P<0.001$).

oxygenation from 3 to 6 h in both the medium ($*P=0.035$) and high ($*P=0.020$) dose HD-exposed groups when compared with the air-exposed group (Figure 2). At 3–6 h post-exposure, there were also statistically significant dose-dependent changes in both oxyhemoglobin and reduced hemoglobin levels when compared with air controls. Oxyhemoglobin levels dose-dependently decreased (Air versus Med $*P=0.03$; Air versus High $*P=0.02$) whilst correspondingly the reduced hemoglobin levels dose-dependently increased (Air versus Med $*P=0.023$; Air versus High $*P=0.033$) (Figures 3 and 4).

Figure 5 shows changes in shunt fraction measured over the course of the study. Shunt fraction is defined as the proportion of blood traveling through the lungs, which remains unoxygenated due to perfusion and ventilation mismatch. The shunt fraction for air-exposed animals was maintained at baseline levels. The shunt fraction of all HD-exposed groups was significantly higher than the air-exposed group from 3 to 6 h (Air versus Low $*P=0.014$; Air versus Medium $**P=0.003$; Air versus High $*P=0.047$).

There were no statistically significant changes in any of the cardiovascular parameters investigated bar CO. Figure 6 shows a statistically significant increase in this parameter in

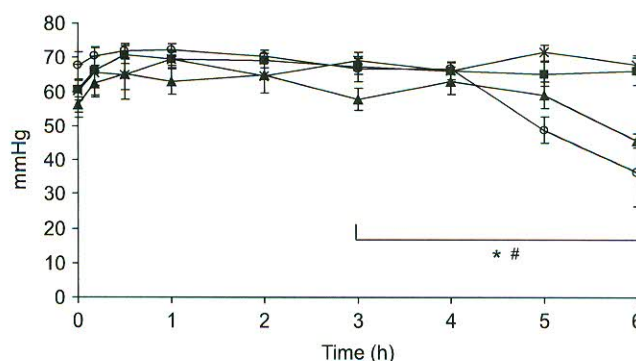


Figure 2. Changes in arterial oxygen tension following exposure to low (■), medium (▲), or high (○) doses of HD, or exposure to air (x). Data are expressed as mean \pm SE. *Significant difference between air and medium-dose HD group over 3–6 h ($P=0.035$). #Significant difference between air and high-dose HD group over 3–6 h ($P=0.020$).

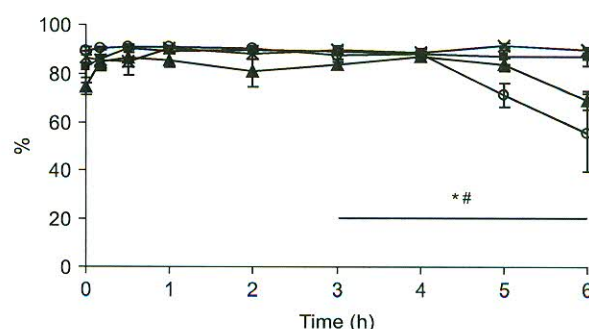


Figure 3. Changes in oxyhemoglobin concentration following exposure to low (■), medium (▲), or high (○) doses of HD, or exposure to air (x). Data are expressed as mean \pm SE. *Significant difference between air and medium-dose HD group over 3–6 h ($P=0.03$). #Significant difference between air and high-dose HD group over 3–6 h ($P=0.02$).

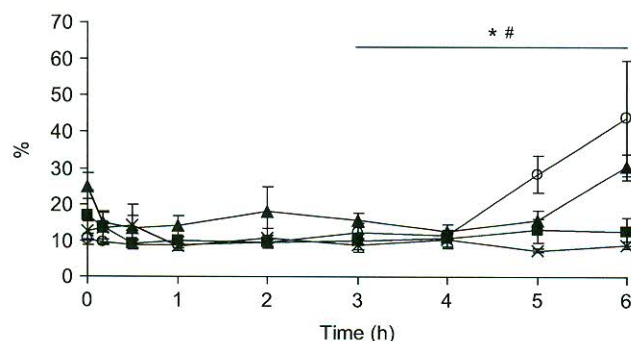


Figure 4. Changes in reduced hemoglobin concentration following exposure to low (■), medium (▲), or high (○) doses of HD, or exposure to air (x). Data are expressed as mean \pm SE. *Significant difference between air and medium-dose HD group over 3–6 h ($P=0.023$). #Significant difference between air and high-dose HD group over 3–6 h ($P=0.033$).

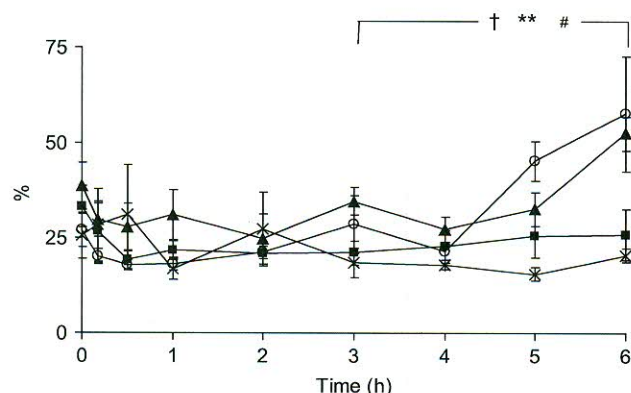


Figure 5. Changes in shunt fraction (%QsQt) following exposure to low (■), medium (▲), or high (○) doses of HD or exposure to air (x). Data are expressed as mean \pm SE. †Significant difference between air controls and low-dose HD group over 3–6 h ($P=0.014$). **Significant difference between air controls and medium-dose HD group over 3–6 h ($P=0.003$). #Significant difference between air controls and high-dose HD group over 3–6 h ($P=0.047$).

the medium-dose HD-exposed group when compared with air controls from 3 to 6 h ($*P=0.016$).

Figure 7 shows a statistically significant decrease in the arterial base excess of the high-dose HD-exposed group when compared with air controls from 3 to 6 h ($*P=0.021$). There was also a statistically significant dose-dependent decrease in arterial sodium bicarbonate levels in HD-exposed animals when compared with air controls 0–3 h post-exposure (Air versus Low $*P=0.028$; Air versus Medium $*P=0.016$; Air versus High $**P=0.006$; data not shown). This was not reflected by a change in the pH of the medium and high-dose HD-exposed animals, which remained stable throughout and not statistically significantly different from air-exposed controls.

Of the hematological parameters investigated, for example, WBC count, red blood cell counts, hematocrit, there were no significant differences between HD-exposed groups and air controls over time.

Figure 8 shows the differential WBC count in terminal lavage fluid. Air-exposed animals had a normal cell population

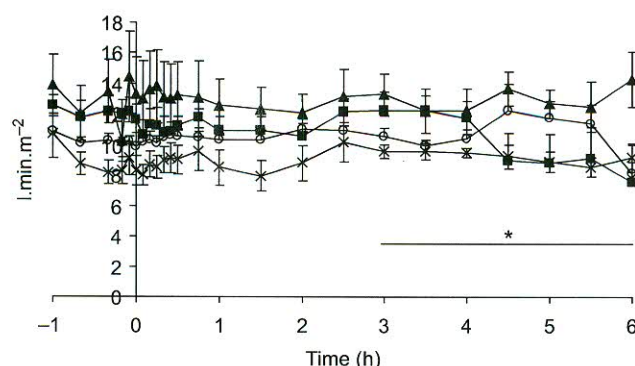


Figure 6. Changes in cardiac output following exposure to low (■), medium (▲), or high (○) doses of HD or exposure to air (x). Data are expressed as mean \pm SE. *Significant difference between air controls and medium-dose HD group over 3–6 h ($P=0.016$).

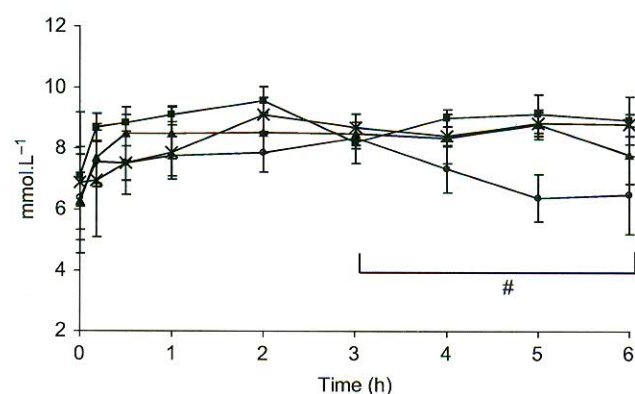


Figure 7. Changes in arterial base excess following exposure to low (■), medium (▲), or high (○) doses of HD or exposure to air (x). Data are expressed as mean \pm SE. #Significant difference between air controls and high-dose HD group over 3–6 h ($P=0.021$).

within the lungs, the majority of the cells being alveolar macrophages and <5% neutrophils. A small dose-dependent neutrophilic influx occurred following HD exposure but this was not found to be statistically significant.

Urine adducts

There was an increase in the concentration of MSMTESE seen over the course of 6 h in all HD-exposed groups. Levels of metabolite of ~ 0.2 ng/mL were detected as early as 2 h post-exposure in the high-dose HD-exposed group (Figure 9). The apparent peak at 5 h post-exposure in the high-dose HD-exposed group results from an individual animal that died shortly after this sample had been taken. This one increased data point (5.2 ng/mL for this individual) accounts for the larger variation seen within this group at this time point.

Cytokine analysis

No changes were seen in the plasma samples for any of the cytokines tested. Thus, only lavage sample analysis is reported here. There were no statistically significant differences between the experimental groups in concentrations

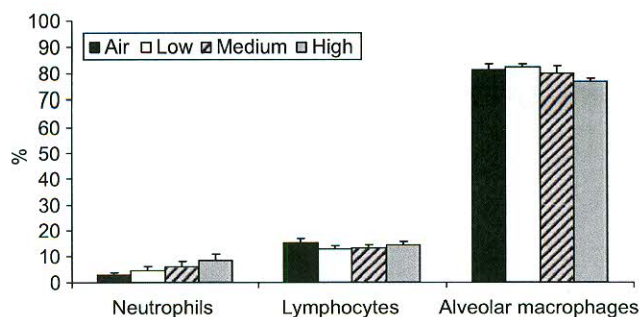


Figure 8. Terminal lavage fluid differential white blood cell (WBC) count following exposure to low (□), medium (▨), or high (▩) doses of HD, or exposure to air (■). Counts were performed using a saline-diluted sample of BAL fluid (1:5 with 0.9% w/v sodium chloride) taken at the end of experiment. Data are expressed as mean \pm SE.

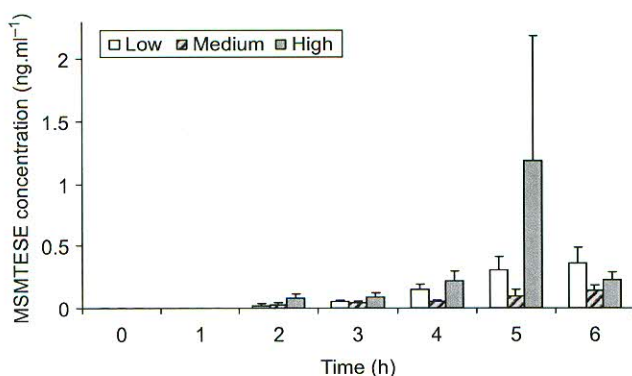


Figure 9. Concentrations of MSMTESE in the urine following exposure to low (□), medium (▨), or high (▩) doses of HD ($n=5$ for each group, except for the high-dose HD-exposed group at 6 h where $n=4$ due to animal death). Data are expressed as mean \pm SE.

of interleukin-6 (IL-6), tumor necrosis factor alpha (TNF- α), leukotriene B₄ (LTB₄), matrix metalloproteinase 2 (MMP-2), IL-8, or IL-1 β . However, small dose-dependent increases in IL-8 and IL-1 β were observed in the HD-exposed groups (Figure 10).

Lung wet weight to body weight ratio (LWW:BW) and histopathology

There was no statistically significant change in LWW:BW ratio or lavage fluid protein concentration between any of the HD-exposed groups and air-exposed animals.

Examination of the tracheal epithelium at postmortem revealed a dose-dependent increase in severity of HD-induced damage (Figure 11). Air-exposed trachea (A) showed intact ciliated tracheal epithelium and normal lamina propria and submucosal structures. Exposure to both low-dose (B) and medium-dose HD (C) gave rise to edema in the lamina propria and mild inflammatory cell (neutrophil) infiltration throughout the mucosa and submucosa. Infiltration was more marked in the medium-dose group. Both groups had apparent blood vessel congestion and areas of nuclear pyknosis. In addition to the above reported effects, exposure to high-dose HD (D) also caused localized sloughing and loss of tracheal

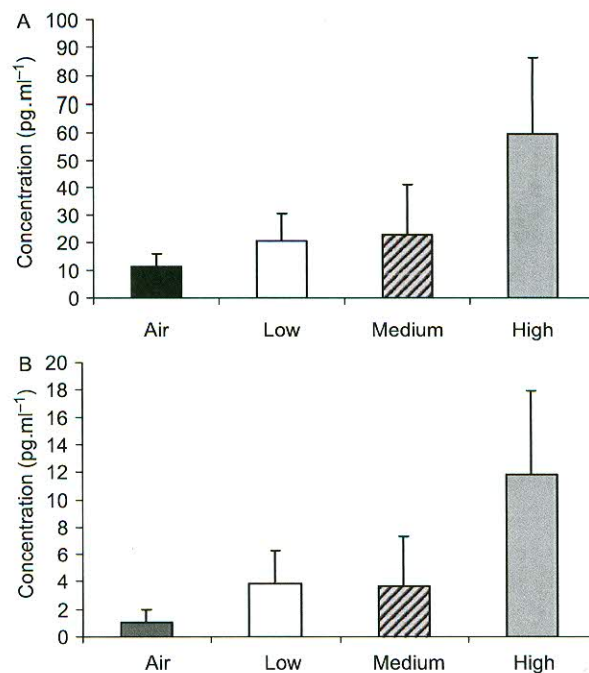


Figure 10. Concentrations of IL-8 (A) and IL-1 β (B) in BAL fluid following exposure to low (□), medium (▨), or high (▩) doses of HD, or exposure to air (■). Data are expressed as mean \pm SE.

epithelium. Damage within the lower parts of the airways was found to be mild. Most relevant were the necrosis of the bronchiolar epithelium, the presence of cellular debris in the bronchiolar region, and evidence of septal and perivascular edema. Plugs of inflammatory cells were found throughout and were found to contain mostly neutrophils.

Respiratory parameters

No statistically significant differences were found between any of the HD-exposed groups and air controls for any of the respiratory parameters investigated, for example, minute volume and respiratory rate. It is likely that 6 h is too soon after HD exposure for these parameters to be significantly affected and that extending the investigation to 12 h may provide considerably greater effects.

Discussion

The mechanism by which HD acts in the lung remains poorly understood. Since the majority of deaths associated with HD exposure have a degree of pulmonary involvement, there is a requirement to further elucidate the underlying mechanisms of HD-induced lung injury. The aim of the study reported here was to investigate the effects of a range of doses of HD vapor exposure directly to the lung in a reliable and quantitatively reproducible manner. This study has demonstrated stable physiology and pathology in air-exposed animals over 6 h, giving us confidence that this is an appropriate model in which to investigate the toxicology of inhaled HD.

Using a novel exposure system (previously described) with which we exposed the lung directly to HD vapor, we

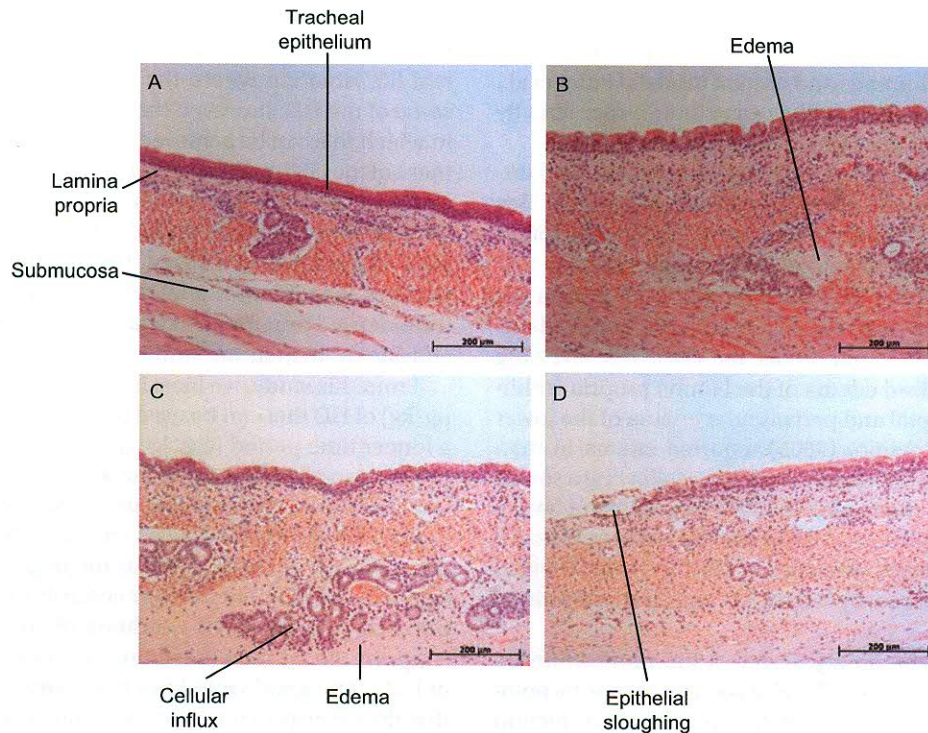


Figure 11. Tracheal epithelium following exposure to HD (original magnification 100 \times). (A) Air control, (B) low-dose HD, (C) medium-dose HD, and (D) high-dose HD.

produced highly consistent, reproducible exposure results and achieved a high degree of accuracy in attaining the target inhaled doses.

Following exposure to inhaled HD, there was a symptom-free period of a few hours with little change occurring in any of the physiological parameters measured before 4 h post-exposure. This is in agreement with studies that have reported the effects of inhaled HD poisoning in man (Papirmeister et al., 1991; Balali-Mood and Hefazi, 2006). Exposure to increasing doses of HD caused changes in a number of physiological parameters by 6 h. In our model, this was seen as a decrease in arterial oxygenation and oxyhemoglobin levels, a corresponding increase in reduced hemoglobin levels, as well as an increase in shunt fraction and CO in HD-exposed animals. HD exposure causes dose-dependent damage to the lung resulting in sloughing of the tracheal epithelium. Cell debris travels deeper into the airways blocking smaller bronchi. Consequently, this causes impaired gas exchange creating a mismatch between ventilation of the lungs and perfusion. This results in an increase in the proportion of unoxygenated blood traveling through the lungs (seen as an increased shunt fraction) and is observed as a subsequent decrease in arterial oxygenation. A dose-dependent reduction in oxyhemoglobin levels confirms the increased proportion of unoxygenated blood, as does the increase in reduced hemoglobin levels. The increased CO reflects the increased workload of the heart attempting to compensate for the reduced arterial oxygenation in the HD-exposed animals.

In the HD-exposed groups, significant decreases were also seen in arterial base excess and arterial sodium bicarbonate

levels, but not in pH. A decrease in base excess but without subsequent change in pH indicates that HD-exposed animals are displaying signs of respiratory acidosis. As a result of HD exposure, the liver is unable to deal with a surplus of H^+ ions and these bind to bicarbonate ions to form carbonic acid and in turn CO_2 and water. This leads to a decrease in the levels of bicarbonate ions (seen as decreasing arterial sodium bicarbonate levels) and so, base excess. The pH is maintained by the renal system. This suggests that the HD-exposed animals become acidotic, but not acidemic, over the course of the study. These results, taken together with the histopathological findings, reflect a similar ALI to that reported in man following inhaled HD exposure. A study of Iranian victims from the Iran-Iraq conflict (Kehe et al., 2009) found significant lung symptoms with 67% of the patients studied having altered blood gas composition. Of these patients, 50% were hypoxic and 17% acidotic. This further supports our model as being appropriate for the study of inhaled HD poisoning. Kehe and Szinicz (2005) also reported the acute effects of moderate HD poisoning in man. Effects include tracheobronchitis, painful and hacking coughing, and formation of pseudomembranes. Detachment of the pseudomembranes and resulting obstruction of the lower airways was also reported. Similarly, in animals exposed to the high dose of HD we found localized sloughing of the tracheal epithelium and cellular debris in the bronchiolar region.

Although not found to be significant, it is thought that the dose-dependent increase in the levels of IL-8 and IL-1 β in the BALF reflects the involvement of cytokines in chemically induced ALI (Emad and Emad, 2007). A small influx of

neutrophils was also seen following inhaled HD exposure. Accumulation of neutrophils in air spaces is consistently seen in ALI in both humans and animal models (Pittet et al., 1997) and it is postulated that they contribute either directly or indirectly to lung injury (Wiener-Kronish et al., 1990).

In our model, inhalation of HD induces upper airway damage, which is most apparent in the trachea and bronchi. This is supported by the findings of Allon et al. (2009) who found sloughing of the tracheal epithelium, necrosis, edema, and blood congestion in the lamina propria in their guinea pig model of inhaled HD-induced ALI. Mild lower airway damage was also apparent in our model, the pathology revealing small areas of localized edema of the lamina propria within the trachea, and septal and perivascular edema of the lower airways. Kehe and Szinicz (2005) reported edema in man both in the upper and in the lower airways following a severe exposure. A lack of change in the LWV:BW ratio and lavage protein concentration seen at 6 h in our HD-exposed animals is likely due to the very acute nature of this time course, which is a limitation imposed by use of an anesthetized animal model.

Of interest was the ability to detect the monosulfoxide β -lyase HD metabolite, MSMTESE at such an early time point post-inhaled HD exposure (from 2 h). This early production may provide a valuable diagnostic tool in identifying inhaled HD exposure, provided samples are taken at an appropriate time post-exposure. MSMTESE and 1,1'-sulfonylbis[2-(methylsulfinyl)ethane] (SBMSE) have been previously used as diagnostic tools to identify exposure to HD following accidental (predominantly) cutaneous exposure (Black and Read, 1995a,b; Barr et al., 2008) and may now also have utility in the diagnosis of poisoning following inhalational exposure. Observation of a large, sudden increase in MSMTESE levels shortly before death (as seen in one animal in the high-dose group) may also provide a valuable prognostic tool in assessing the degree of HD damage. MSMTESE, unlike thiodiglycol and its sulfoxide derivative (Balali-Mood and Hefazi, 2005), is an unequivocal marker of HD exposure and can be reduced to thioether derivatives for analysis by GC-MS (Black and Read, 1988). SBMSE, the bis-sulfoxide β -lyase HD metabolite, could not be detected in any of the samples. This was due to a large amount of interfering material being extracted from the pig urine. This is not observed in human urine.

The time course of effects is perhaps more acute in our model than that seen in man. This could be explained in two ways. First, for practical reasons, to achieve the chosen inhaled doses a high concentration was delivered over a short exposure duration (~10 min). This enabled us to cause an injury within the 6-h study period. This may not reflect the real life situation where man is likely to be exposed to a lower concentration for a longer period of time due to the persistency of HD. Delivery of a high dose over a short period may result in a more severe injury. Second, our model was designed to represent human exposure, bypassing the URT and exposing directly to the lung, as, in times of stress or exercise (e.g. military personnel on the battlefield or civilians running to escape a scene of chemical release), man

breathes more via the mouth than the nose, increasing the load in the deep lung. This does not completely mimic the real life situation where the nasal passages would absorb some of the HD; however, there is no existing animal model in which this can be achieved. It is possible that the involvement of the URT may remove a percentage of the HD inhaled and so a reduced dose reaches the lung. This may result in a longer asymptomatic period before an effect on oxygenation, for example, is realized. Despite these differences, the changes seen in our model reflect those seen in man but in a condensed timeframe. It will be important to take this into consideration in future studies.

From this study, we have identified an inhaled dose (100 $\mu\text{g}/\text{kg}$) of HD that can be used to study the effects of HD over a longer time period (e.g. 12 h). We conclude that this dose produces a significant and consistent ALI, which is severe but not fatal up to 6 h and against which the efficacy of candidate therapeutic strategies can be investigated. The low dose (60 $\mu\text{g}/\text{kg}$) was rejected as the degree of injury was not significantly different from air controls in terms of oxygenation and pathology. The condition of the animals at 6 h also supports the opinion that animals exposed to the high dose of HD (150 $\mu\text{g}/\text{kg}$) would not have survived out to 12 h and that the severity of the injury was too great given that two of the animals exposed to this dose died before 6 h. The two animals were unable to clear obstructions within the ET tube by coughing due to the inflexible nature of the PTFE liner, which is another limitation of the model.

It is hoped that investigation of the effects of inhaled HD out to 12 h will help to further elucidate the underlying mechanisms of action and identify therapeutic targets. These might then be compared with other chemicals causing an ALI, for example, phosgene (Brown et al., 2002), in order to define whether any common pathways exist in the injury process. This might identify new generic candidates for the treatment of chemically induced ALI whose efficacy can then be tested in this robust model.

Conclusions

Exposure to inhaled HD in our terminally anesthetized spontaneously breathing large white pig model caused significant changes in shunt fraction from 3 to 6 h post-exposure. Animals were increasingly hypoxic and showed signs of respiratory acidosis. Pathology revealed necrosis and erosion of the tracheal epithelium in medium and high HD-exposed groups as well as localized sloughing of the epithelium. These findings are consistent with those seen in the early stages of ALI. Additionally, MSMTESE, a monosulfoxide β -lyase metabolite of HD, was detected in the urine from 2 h post-exposure. MSMTESE has previously been used as a diagnostic marker of HD exposure (following predominantly cutaneous exposure) and may now have utility in the diagnosis of poisoning following inhalational exposure. We recommend that our model is an appropriate one to investigate the efficacy of promising therapeutic candidates in the treatment of chemically induced ALI.

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Declaration of interest

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Preliminary Studies of Sulphur Mustard–Induced Lung Injury in the Terminally Anesthetized Pig: Exposure System and Methodology

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ABSTRACT Although normally regarded as a vesicant, inhalation of sulphur mustard (HD) vapor can cause life-threatening lung injury for which there is no specific treatment. Novel therapies for HD-induced lung injury are best investigated in an *in vivo* model that allows monitoring of a range of physiological variables.

HD vapor was generated using two customized thermostatically controlled glass flasks in parallel. The vapor was passed into a carrier flow of air ($81 \text{ L} \cdot \text{min}^{-1}$) and down a length of glass exposure tube (1.75 m). A pig was connected to the midpoint of the exposure tube via a polytetrafluoroethylene-lined endotracheal tube, Fleisch pneumotachograph, and sample port. HD vapor concentrations ($40\text{--}122.8 \text{ mg} \cdot \text{m}^{-3}$) up- and downstream of the point of exposure were obtained by sampling onto Porapak absorption tubes with subsequent analysis by gas chromatography-flame photometric detection. Real-time estimates of vapor concentration were determined using a photo-ionization detector. Lung function indices (respiratory volumes, lung compliance, and airway resistance) were measured online throughout.

Trial runs with methylsalicylate (MS) and animal exposures with HD demonstrated that the exposure system rapidly reached the desired concentration within 1 min and maintained stable output throughout exposure, and that the MS/HD concentration decayed rapidly to zero when switched off.

A system is described that allows reproducible exposure of HD vapor to the lung of anesthetized white pigs. The system has proved to be robust and reliable and will be a valuable tool in assessing potential future therapies against HD-induced lung injury in the pig.

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KEYWORDS Endotracheal Tube; Inhalation; Lung Compliance; Lung Resistance; Methylsalicylate (MS); Minute Volume; Model Development; Pig; Porcine; Sulphur Mustard (HD) Vapor; Bis(2-chloroethyl)sulphide

INTRODUCTION

Sulphur mustard[bis(2-chloroethyl)sulphide; HD] is an alkylating agent whose first use as a chemical warfare agent was in the trenches during World War 1 (WWI) at Ypres in 1917 (Prentiss 1937). HD was sometimes known as the “king of the battle gases” and was responsible for in excess of 120000 British gas casualties. It has been used in numerous locations across the world since that time (Stockholm International Peace Research Institute [SIPRI] 1971). During the Iran–Iraq conflict it was also widely used resulting in over 100,000

medical casualties (Hefazi et al. 2005; Pechura and Rall 1993). Its effectiveness is due, in part, to its low threshold of effect, with HD causing incapacitation at concentrations as low as $0.0065 \text{ mg} \cdot \text{L}^{-1}$ following 60 min of exposure (Prentiss 1937).

Although commonly referred to as "mustard gas," it is a liquid at room temperature (boiling point 217°C , freezing point 14.4°C) and both the liquid and vapor phases cause its characteristic vesicant properties (Marrs et al. 1996). The major effects of HD are ocular injury (conjunctivitis, temporary blindness) and dermal injury via the percutaneous route but of equal importance are the respiratory effects. These range from an unproductive cough following mild exposure to pulmonary edema and respiratory failure, often resultant from the infection that can arise from damage to the airway epithelium (Kehe and Szinicz 2005). The majority of deaths from HD poisoning during WWI had some degree of pulmonary involvement (Prentiss 1937).

Military personnel, in the absence of detection equipment, may be unaware that they have been exposed to HD vapor as the odor detection threshold is reported to be quite high ($0.6 \text{ mg} \cdot \text{m}^{-3}$; Pechura and Rall 1993), the vapor would be invisible to the human eye, and there is a delay in the onset of symptoms for at least 4 h. Therefore, personnel could inhale a lethal quantity of HD vapor without realizing they are being exposed. HD is highly persistent so there is also a high risk of vapor "off-gassing" from contaminated clothing, equipment, etc., when in a "clean" environment. If HD were to be used in a terrorist attack, the civilian population would also be unfamiliar with the odor and lack suitable respiratory protection.

At the present time no specific treatment exists for HD poisoning and so it remains an important requirement to find an effective therapy; therefore, there is a need to develop suitable models in which the injury can be reliably reproduced. This in turn will permit further studies on the mechanism of action of HD and also the effects of any potential treatments. The effects of HD have been studied *in vitro* but the biochemical and immediate effects of HD lung injury are not fully understood. To date, a small number of studies have been carried out to investigate HD-induced lung injury in whole animal models. An early study by Winternitz and Finney (1920) looked at the pathology of dog lungs exposed to HD vapor during WWI. Although no details of exposure concentrations were given, the authors demonstrated that exposure to HD vapor resulted in the formation of a pseudomembrane that covered the interior surfaces of the tracheobronchial tree. Cameron et al. (1946) then demonstrated that HD is readily absorbed across the respiratory tract, including the nasal membrane. Box and Cullumbine (1947) described the effects of HD vapor on mice and estimated the LC_{50} to be $101 \text{ mg} \cdot \text{m}^{-3}$ following a 10-min exposure.

For agents such as HD, which cause extensive damage to the upper respiratory tract (URT), it is difficult to extrapolate the results of studies carried out in small animals to man. Since rodents are obligate nasal breathers, mustard injury in rodents tends to be more severe in the URT, whereas in humans, who are not obligate nose breathers, it is the lower respiratory tract that is most affected. It is therefore essential to find a species more directly comparable to man so that any data derived can be extrapolated with greater confidence.

The large white female pig (approximately 50 kg) was chosen for this study as it is a well-studied and understood species. The lung physiology is comparable to that of man (Cramer et al. 1994; Getty 1975). The size of the animal also means that conventional human intensive care unit (ICU) equipment

can be utilized with the pig because of similarities in the tracheobronchial and vascular architectures. This makes the large white pig a more realistic model for the investigation of therapy for human lung injury. The pig has been widely used as a model of acute lung injury caused by inhaled agents such as chlorine (Gunnarson et al. 1998) and phosgene (Brown et al. 2002), by bacterial infection (Dehring et al. 1983), and by physical trauma (Thörne et al. 1989). The use of the pig also allows the URT to be bypassed, enabling the effects of HD delivered directly to the lung to be investigated. This better reflects human exposures as, unlike smaller animal models (e. g., rat, mouse, guinea pig), which are obligate nasal breathers, man breathes via the mouth particularly during periods of stress or exercise (Brown et al. 2006). Delivering HD vapor direct to the lung would also be extremely difficult in a smaller animal model such as the rat because of the need for specialist equipment and the technical challenge of intubation. Finally, our laboratories have established expertise in using this particular animal model (Brown et al. 2002, 2006).

The aim of the work described here was to develop a system for exposing the anesthetized large white pig to HD vapor direct to the lung, bypassing the URT, in a reliable and quantitatively reproducible manner. This will facilitate the future development of therapeutic strategies against HD-induced lung injury.

METHOD

All animal work described here was carried out in accordance with the UK Animals (Scientific Procedures) Act of 1986, and approved by the Ethical Review Committee of Dstl, Porton Down.

The system was assembled and operated within a charcoal filtered fume cupboard with a face velocity greater than $0.5 \text{ m} \cdot \text{s}^{-1}$ (Fig. 1a). HD vapor was generated from two custom vapor generators operated in parallel (Fig. 1b). These are thermostatically controlled water-jacketed glass flasks, the internal temperature of which can be adjusted by changing the temperature of the circulating water. Each vapor generator contained approximately 11 mL of neat HD. The available surface area for evaporation was increased by the use of pleated filter paper (total surface area in each generator approximately 570 cm^2). Changing the temperature of the circulating water changed the output of mustard vapor. To maintain the stability of the HD, the generators were ventilated either with dry nitrogen (to prevent reaction of mustard with water vapor or oxygen between exposures) or, for animal exposure, dry air. Our previous experience has shown that the mustard remains stable for several months under these conditions, and that sufficient mustard is contained within the generators for at least 20 exposures. Each generator was also fitted with a simple pressure relief device consisting of a bubbler filled with mercury to a depth of 1 cm to prevent accidental overpressure.

The generated mustard vapor was passed from the generators into either a charcoal bed, or by switching a tap, into a carrier flow of $81 \text{ L} \cdot \text{min}^{-1}$ passing down a 1.75-m length of 7.6-cm-diameter glass exposure tube. This high carrier flow was necessary to satisfy the animal's peak inspiratory and expiratory flows. All tubing upstream of the exposure tube was warmed using electrothermal heating tapes to prevent condensation of mustard on the internal walls. All parts of the exposure system were made from polytetrafluoroethylene (PTFE), glass, aluminium, or steel; plastics and rubber were not used as

mustard can absorb into these materials and then subsequently be slowly released for many hours. The apparatus contained static mixers to prevent the mustard vapor from plumbing down the middle of the tube. Charcoal filters were used to scavenge mustard vapor after passage through the exposure apparatus at all times.

To reduce risk, the apparatus was firstly characterized using an HD simulant, methyl salicylate (MS), to determine the system characteristics prior to exposing animals.

For animal exposures to HD, the anesthetized juvenile large white pig was connected while in a supine position to the exposure tube via a PTFE-lined endotracheal (ET) tube, a size 2 Fleisch pneumotachograph, and a sample port (Fig. 2) following a 1-h baseline period. The kit was designed such that the pig's ET tube was located inside the fume cupboard while the pig's body remained outside, at a 90-degree angle to the front of the fume cupboard. Air flow rates through the generators were kept constant at $3 \text{ L} \cdot \text{min}^{-1}$. The apparatus was initially set up to deliver the required inhaled dose over a 10-min exposure assuming an animal with a minute volume of $7.0 \text{ L} \cdot \text{min}^{-1}$ and a body weight of 50 kg.

When the weighed animal arrived in the exposure facility, it was connected to the exposure apparatus and respiratory monitoring system. Minute volume and other lung function variables were monitored in real time using eDacq (EMMS Data ACquisition) software (Infodisp, UK) throughout, and subsequent to, the exposure. Effort was made to maintain the minute volume between 6 and $8 \text{ L} \cdot \text{min}^{-1}$ by controlling the dose of anaesthetic. The inhaled dose was then estimated every minute during the exposure, and the exposure terminated when the inhaled dose reached the desired value. Thus, while the

exposure duration was planned to be 10 min, in practice it varied between 6.5 and 12.6 min to account for variations in the animal's minute volume from the planned norm of $7 \text{ L} \cdot \text{min}^{-1}$.

Mustard vapor concentrations up- and downstream of the point where the animals were exposed were obtained by sampling at a constant rate onto Porapak absorption tubes, which were then analyzed by solvent (hexane) elution and quantitative gas chromatography-flame photometric detection (GC-FPD). For this, a midpolar capillary column was used and the mobile phase was helium carrier gas at a flow rate of $2.5 \text{ mL} \cdot \text{min}^{-1}$. This analysis was also used for the MS characterization absorption tubes but these were eluted with propan-2-ol. From the upstream results inhaled HD dose was calculated using the animals total inhaled volume over the exposure period and the animal's weight. Real-time estimates of upstream HD vapor concentration could also be determined during exposure using a photo-ionization detector (miniRAE 2000 [Rae Systems Inc.] or TVOC [Ionscience Ltd.]).

RESULTS

Characterization with Methyl Salicylate

Vapor generator output, as determined by upstream Porapak sample tube analysis, increased with increasing water bath temperature (Fig. 3a, $r^2 = 0.997$). In addition, the miniRAE gave significant correlation ($r^2 = 0.988$) with the sample tubes upon analytical determination (Fig. b). Further trials

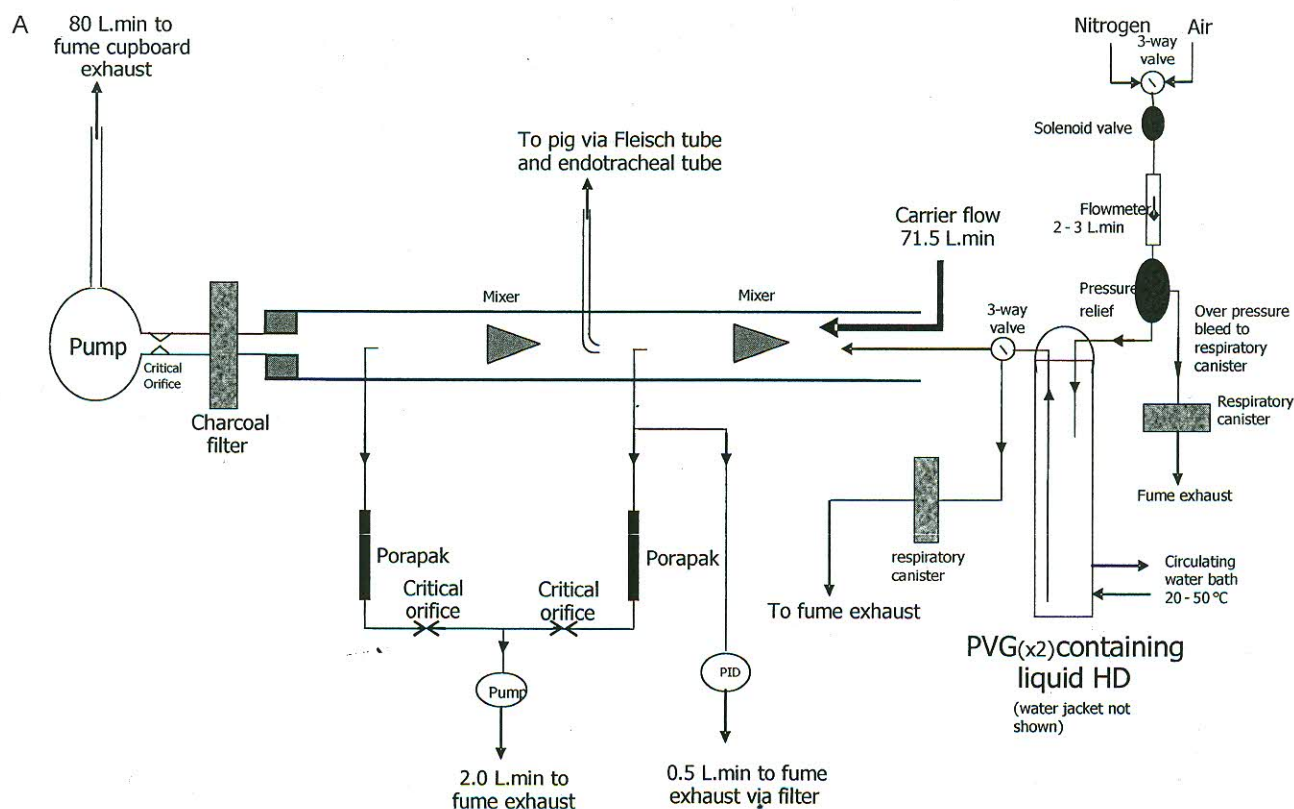


FIGURE 1 A: Schematic showing exposure apparatus setup and relative position of HD vapor generators for the generation of HD vapor and delivery to pig. B: Single HD vapor generator in more detail.

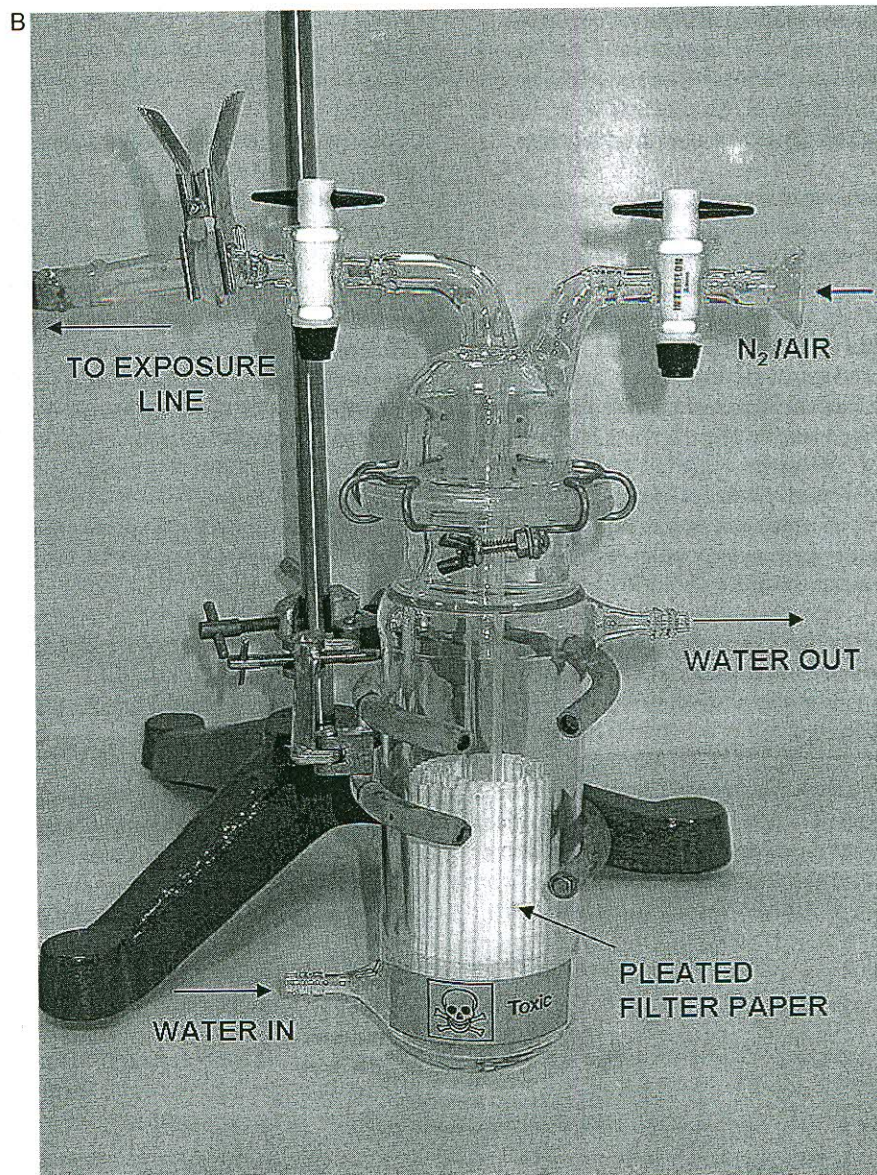


FIGURE 1 Continued

(Fig. 3c) demonstrated that a stable MS concentration could be achieved for at least the required exposure duration (10 min). MS condensation was seen on the first mixer at generation temperature = 50°C, limiting the maximum temperature to 45°C for subsequent trials.

The results obtained with MS showed that the apparatus was working as expected, so further experiments were carried out using HD.

Characterization with HD and Animal Exposures

Nine HD vapor trials demonstrated that stable and predictable mustard concentrations could be achieved. The effect of bath temperature on mustard concentration from both the trial runs and animal exposures ($n = 15$) is shown in Figure 4. As the water bath temperature was increased, so the HD concentration increased ($r^2 = 0.947$, slope significantly nonzero

$[p < 0.0001]$ for the dummy run; $r^2 = 0.66$, slope significantly nonzero $[p < 0.0002]$ for the animal exposures). Animal exposures showed more variability compared with dummy runs. Similar to the results seen with MS, HD vapor concentration increased with temperature over the range of 25.0 to 45.0°C, producing HD concentrations ranging from 40.0 mg·m⁻³ to 122.8 mg·m⁻³. Figure 5 shows HD vapor concentration, as determined by miniRAE PID, from three animal exposures (one animal per dosing group; exposure time = 10 min). The experiments clearly demonstrated that the target HD concentration was rapidly achieved after switch-on, was maintained with an acceptable level of stability, and rapidly decayed to zero when the HD generators were switched off.

Respiratory Monitoring

The eDacq software successfully measured the many respiratory parameters in real time during the course of each

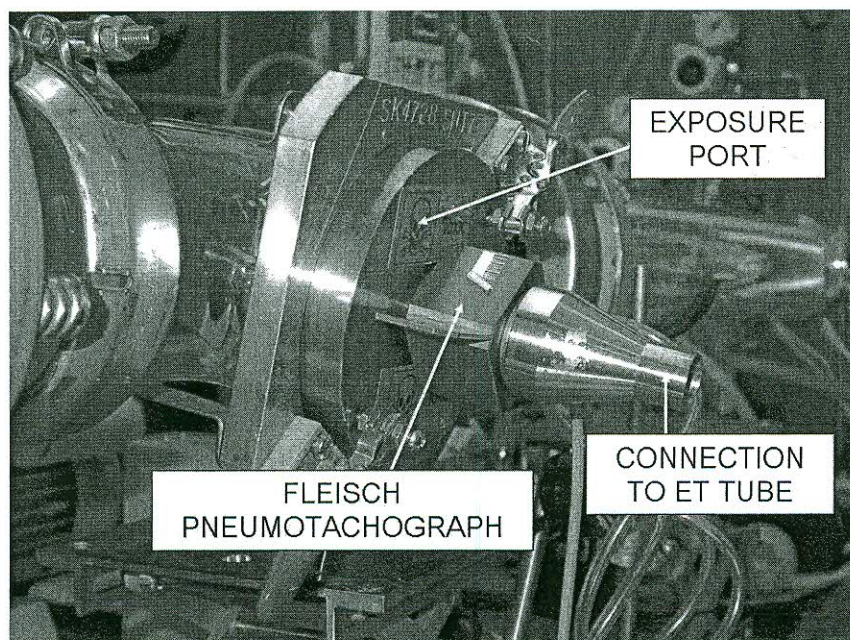


FIGURE 2 HD vapor exposure port and Fleisch pneumotachograph, which allow connection of the animal to the apparatus enabling delivery of HD vapor direct to the lung via the endotracheal (ET) tube.

experiment. Examples of the data produced are illustrated in Figure 6. This data were taken from one animal exposed to $100 \mu\text{g} \cdot \text{kg}^{-1}$ HD vapor. The graphs in Figure 6 are typical of what was observed for the majority of the animals across all groups. After an initial 1-h baseline period, the measurements tended to remain stable until approximately 5 h postexposure when it became possible to detect changes. Analysis of these changes will be reported in a separate publication.

The eDacq software displayed measurements of minute volume (and other respiratory variables) in real time. This information could be used to estimate inhaled dose as the exposure progressed, and allowed the exposure to be terminated when the desired inhaled dose was approached. This technique was successfully used to control inhaled doses so that the achieved doses were close to the target doses, and also to minimize the interindividual variation in inhaled dose (see Table 1a and b).

DISCUSSION

The results obtained in this preliminary study have shown the vapor-generating apparatus to be capable of delivering reproducible and stable concentrations of HD to the lung

of the juvenile large white pig for at least 10 min. The PVG is well characterized as a generation system, having been used in-house to generate a number of agents with a wide volatility range, including nitrogen mustard and nerve agents. The concentration generated is determined by four factors: (i) the surface area of the filter paper, (ii) the temperature at which the generators are set, (iii) the flow rate through the generators, and (iv) the flow rate of the carrier air through the system. The generation system was validated at a number of different temperatures and the results clearly demonstrate that the concentration is achieved quickly, remains stable for the period of exposure, and then returns rapidly to zero when switched off.

The exposure system is a development of that used to expose pigs to phosgene (Brown et al. 2002). The use of eDacq and miniRae allowed greater control over the inhaled dose delivered to the animal. This was advantageous over delivering a set Ct (concentration \times time) since this would have resulted in the animals receiving significantly different inhaled doses due to interindividual variations in individual minute volume and body weight. While it would arguably have been more desirable to adjust the mustard concentration to account for changes in minute volume and maintain a constant exposure duration, this

TABLE 1a Summary of target versus achieved inhaled HD vapor doses during animal exposures

Dose Group	Target Dose ($\mu\text{g} \cdot \text{kg}^{-1}$)	Achieved Dose ($\mu\text{g} \cdot \text{kg}^{-1}$)	% Difference	SEM	n	Achieved Different from Target (t-test)?
A	60	67.2	12.00	4.3	5	No, $p = 0.17$
B	100	100.6	0.60	4.6	5	No, $p = 0.90$
C	150	157.8	5.20	10.0	5	No, $p = 0.48$

Inhaled dose ($\mu\text{g} \cdot \text{kg}^{-1}$) is calculated from the concentration of HD, determined by solvent (hexane) elution from the upstream Porapak tube. Achieved doses were not significantly different from target doses.

TABLE 1b One-way ANOVA and Bonferroni's multiple comparison *t*-test analysis of achieved doses between groups A through C, showing significant differences between groups

Dose Group Comparison (from Table 1a)	Significance Level
A vs. B	$p < 0.05$
B vs. C	$p < 0.001$
A vs. C	$p < 0.001$

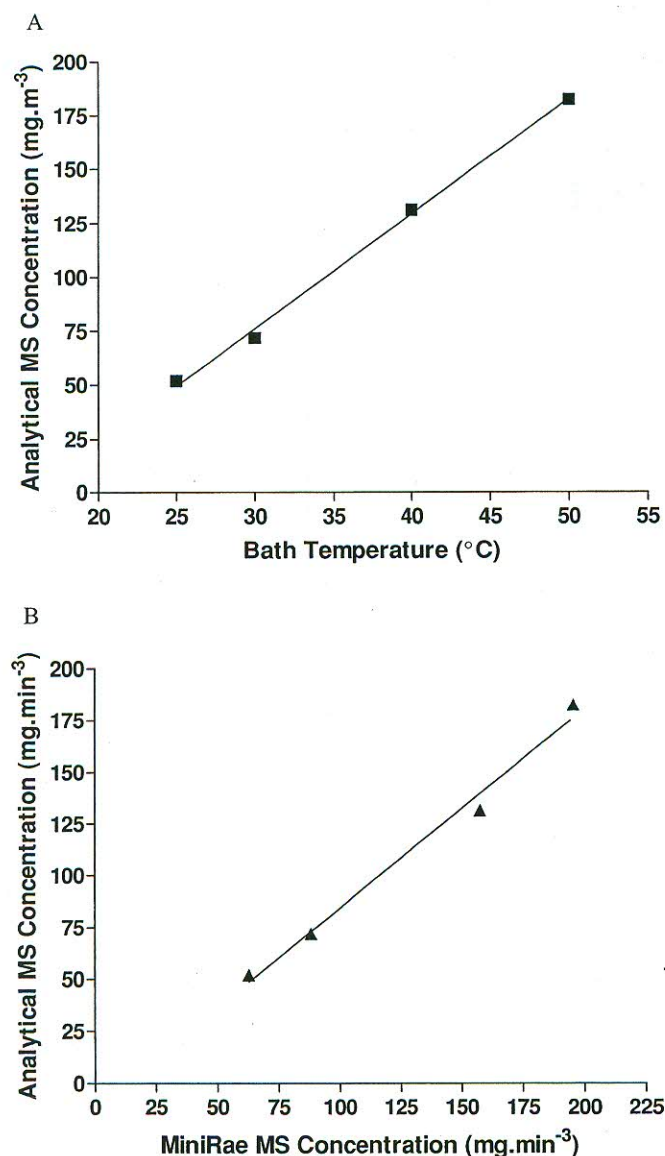


FIGURE 3 A: Concentration of MS vapor produced at increasing water bath temperatures (data analyzed from up stream Porapak tubes). $r^2 = 0.997$, slope $p < 0.001$ from 0. B: Correlation of MS concentration between the miniRAE and Porapak tube data. $r^2 = 0.988$, slope $p < 0.005$ from 0. C: Representative graph showing concentration of MS vapor over time. Stability of MS concentration over one 10-min exposure period shown for system at 40°C (■) and 50°C (▲). MS concentration determined by miniRAE PID.

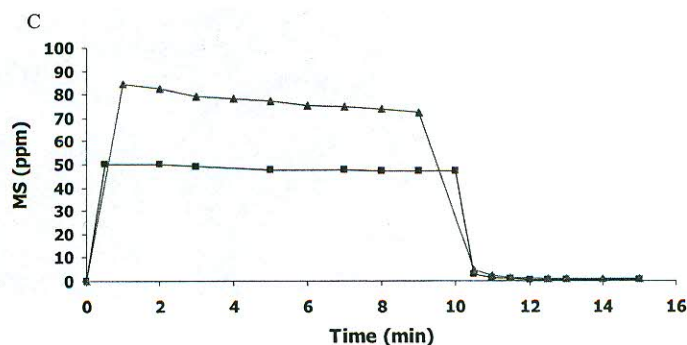


FIGURE 3 Continued

was not feasible due to the design of the vapor generators, which does not permit rapid changes in vapor output.

Minor technical problems were identified and dealt with during the characterization of the equipment. It is important to highlight what they were and how they were overcome for future studies. When generating MS at temperatures = 50°C, condensation of MS was apparent on the static mixer within the exposure tube; therefore, 45°C was the highest temperature used for animal exposures.

The location of the catheter used to measure esophageal pressure (esophageal pressure is believed to be representative of transpulmonary pressure and is used to calculate compliance and resistance) was also of vital importance. Improper placement could result in physiologically unreasonable compliance and resistance values. It is important to ensure that the catheter is sited in the same place in each pig to ensure consistency.

Proper adjustment of the eDacq system was also necessary to prevent heartbeats appearing on the flow signal, which could affect the measured minute volume and rate, potentially giving false readings.

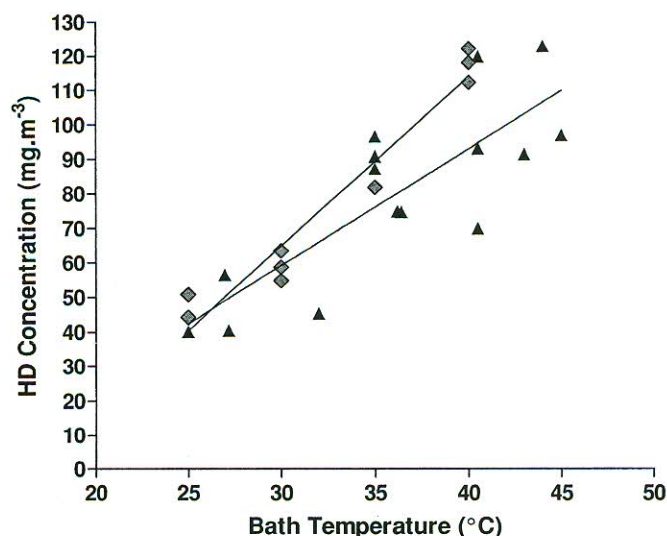


FIGURE 4 Concentration of HD vapor produced at increasing water bath temperatures. Each point represents a dummy run (no animal) (♦) or individual animal exposure (▲) with HD vapor concentration determined by Porapak tube.

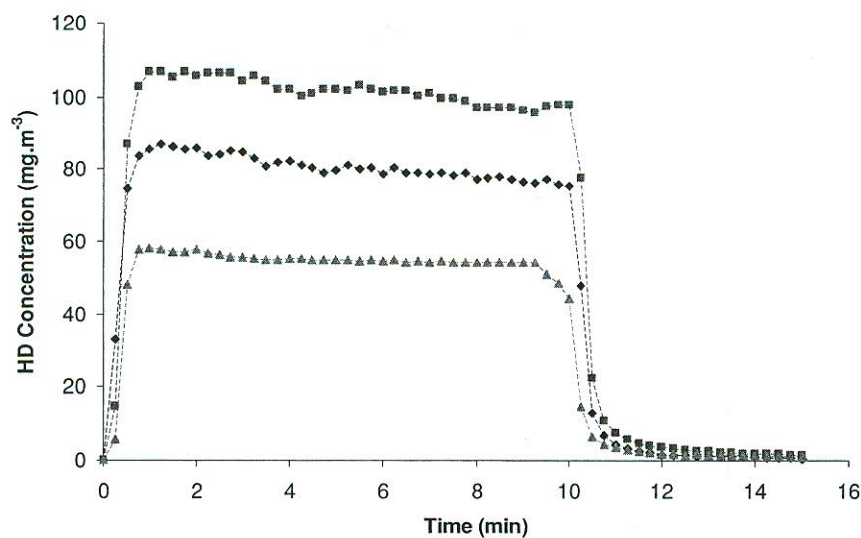


FIGURE 5 Concentration of HD vapor over duration of 10-min exposure. Each line represents an individual animal exposure from a different dosing group. Data obtained from miniRAE PID.

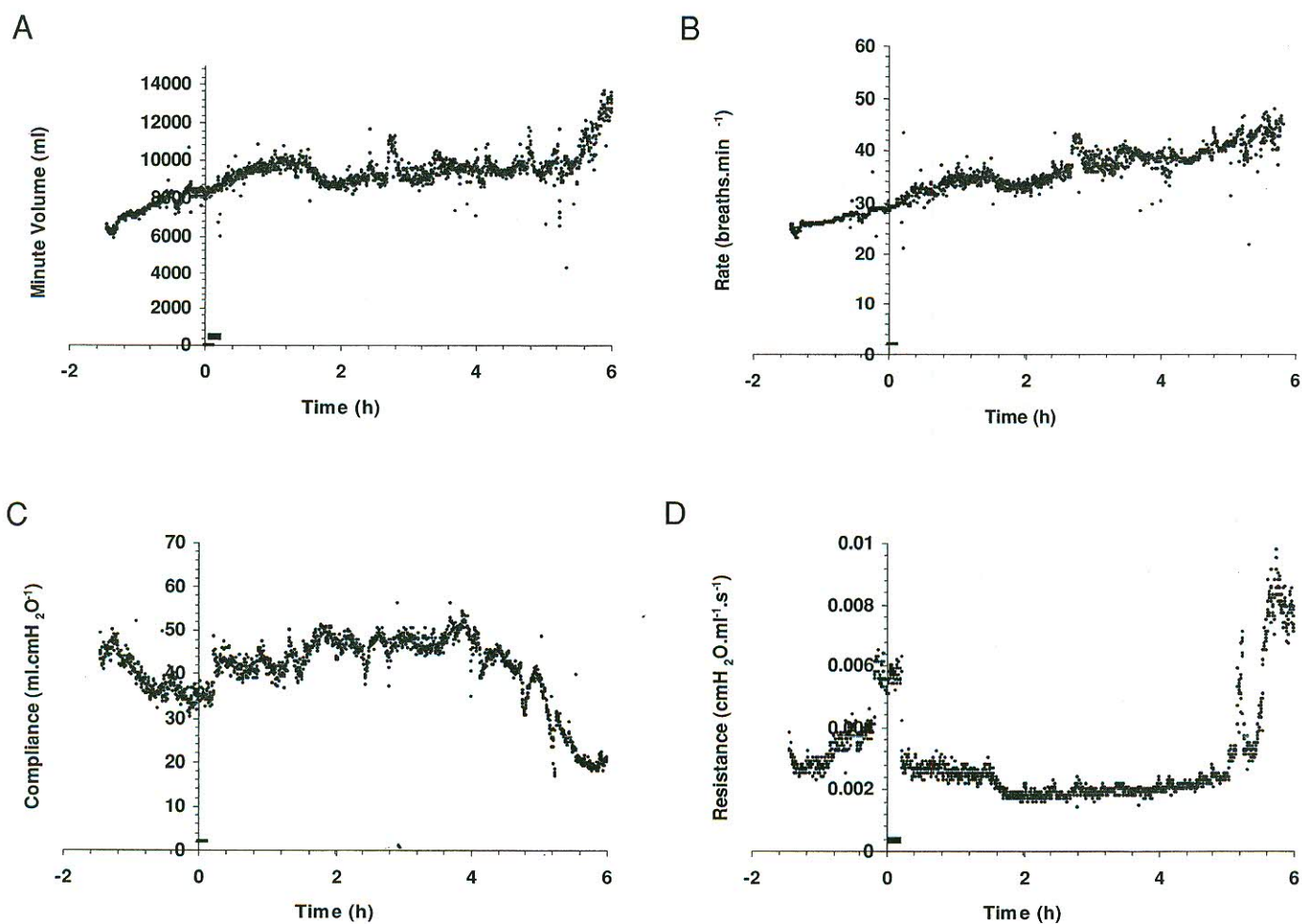


FIGURE 6 Illustrative data showing effect of HD vapor on respiratory parameters; minute volume (A), respiration rate (B), compliance (C), and resistance (D) over time in one animal. Each point represents 15 s of real time data. The black bar (—) indicates the 10-min HD vapor exposure period.

Use of a miniRAE to monitor HD concentration is potentially problematic. Despite excellent performance during the characterization phase and initial animal exposures, with continued use the miniRAE has proved vulnerable to a combination of high HD vapor concentrations and water vapor. Upon deterioration of performance, it was noted that HD had deposited onto the PID lamp and water vapor had leaked past a Teflon sealing washer and corroded the electrode components. An alternative photo-ionization detector, TVOC, has been proposed and will be reported on in a subsequent publication.

There were a number of safeguards in place that allowed safe operation of the apparatus when using HD. These included the mercury pressure relief devices to prevent overpressuring the vapor generators and the use of charcoal filters to prevent HD vapor escaping the exposure apparatus. A real-time hand-held detector (AP2C, Proengin, France) was also used to monitor for the presence of HD vapor in the laboratory. To date, no HD has been detected by this instrument in the laboratory atmosphere. In addition, samples were taken continuously onto tenax absorption tubes from the area in front of the fume cupboard during the exposure period and then again for the duration of the study (up to 6 h). Further samples were taken around the animal and within its thoracic cavity during autopsy. These tubes were then analyzed by GC-FPD with thermal desorption for the presence of HD as described earlier. The maximum amount of HD found on the tubes during and after exposure was 5.9×10^{-7} mg, which does not represent a hazard to the operators. As a further safeguard, staff performing autopsy wore respirators.

The versatility of this system means that it could have potential applications in other areas. Both the sample ports and exposure line have been engineered to be suitable not only for HD vapor, but also if the flow rates are modified for other gases and aerosols.

In conclusion, we have developed a robust, safe, and reliable exposure system capable of delivering reproducible concentrations of HD vapor to the lung of the anesthetized white pig. This system can now be used to examine the effects of HD vapor of a known dose on lung injury over a longer time period and investigate the efficacy of potential treatments in improving outcome following acute lung injury. The versatility of the system also means it may have future applications for exposing other species to HD vapor or other agents with minimal modification.

The eDacq respiratory monitoring software allowed real-time measurement of respiratory parameters to a high degree of sensitivity. Measurements of inhaled volume taken over the period of HD exposure allowed inhaled dose to be calculated. Because of its real-time output, it was also useful to rapidly

detect any changes in the animal, allowing, for example, decisions to be made regarding the doses of anesthetics required.

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